A CONTRIBUTION TO THE KNOWLEDGE

OF THE

PRODUCTS OF THE REDUCTION OF HAEMATIN

By

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For the last two years I have been engaged in work upon the products of the decomposition of blood pigments by means of reducing agents with the view of elucidating the changes undergone by blood pigment within the animal organism.

The following Thesis on this subject is divided into three parts. The first of these consists of a brief introductory sketch of what is known with regard to the katabolism of blood pigment. An historical review of the results obtained by the action of reducing agents upon haematin forms the second part; while the third part contains an account of the results which I have obtained from work on the action of reducing agents upon haematin.

Three different methods of investigation may be adopted for the study of the katabolism of blood pigment. One may either investigate the changes which the pigment undergoes in localised extravasations of blood, or one may study the products of its decomposition which are found in the excreta under both normal and pathological conditions, or finally one may combine with the latter method the investigation of the effects of experimental interference with the functions

functions of the organs believed to be concerned in the breaking down of blood pigment with the view of ascertaining not only the sites of formation of the various pigment derivatives: but also the nature of the chemical changes to which they owe their origin.

These different methods and their results will now be reviewed in order.

The earliest observation of scientific importance on the changes undergone by blood pigment within local extravasations of blood is due to Virchow(1)(1847). He noted the replacement of haemoglobin by a crystalline pigment "haematoidin," and also emphasized its likeness to bile pigment. Haematoidin was subsequently proved to be identical with the bile pigment, bilirubin. Later observers (2, 3, 4) studied the changes undergone by blood pigment when injected into the subcutaneous tissue. Langhans (5) found that in birds the transformation of haemoglobin into bilirubin and biliverdin only required three or four days. Similar experiments on dogs by Quincke (6) indicated that the transformation required about nine days. Another important observation is due to Recklinghausen who found that bile pigment is formed in frogs' blood outside the body within three to ten days. The frog's blood was kept aseptic during

during the transformation.

The formation of bilirubin involves the decomposition of the haemoglobin molecule into the proteid globin and the pigment haematin together with a small amount of other bodies of unknown nature. Finally the iron is withdrawn from the haematin and bile pigment formed according to the equation

$$C_{32}H_{32}N_4O_4Fe + 2H_2O - Fe = 2C_{16}H_{18}N_2O_3$$

Haematin or more probably haemochromogen is found in the first instance along with globin, and has been found occasionally in extravasations. Since oxyhaemoglobin yields on decomposition only about 4·2 per cent of haematin, the pigments arising secondarily are mixed with relatively large quantities of globin and the products of its decomposition. The iron appears partly to enter into loose combination with these albuminous residues to form chemically ill defined pigmented substances which have received the names of "melanin" (Latschenberger)(7) and haemosiderin (Neumann)(8) partly to be found as the free oxide. Latschenberger has described reddish yellow pigments which are apparently intermediate between haematin and bilirubin under the name of choleglobine.

On microscopical examination he found dark orange and yellow spherules about one-fourth the

the size of a red corpuscle. These gave a well marked Gmelin's reaction.

The presence of bile pigment in the placenta is probably also to be ascribed to its formation *in situ*. A large amount of work has since been done upon the changes which blood pigment undergoes after injection into the subcutaneous tissue and blood stream; but more attention has been directed to the effect on bile excretion and to tracing the fate of the iron than to the chemical examination of the pigments formed.

The following brief review may now be given of the pigments found in bile, their sources and mode of formation. I shall only mention those pigments the chemical characters of which are well defined. The two well known bile pigments bilirubin and the first product of its oxidation biliverdin are the only colouring matters found in fresh bile which have been thoroughly investigated. The probability of the existence of certain other bile pigments present in normal bile will be discussed in a later portion of this thesis. It has been already proved that bile pigment may be formed apart from the liver. The question therefore naturally arises as to whether the liver is merely an excretory organ for bile

bile pigment formed elsewhere or whether it is also an organ in which bile pigment is formed. This question has, it is needless to say, been decided in favour of the latter view.

Tarchanoff[15] and subsequently Vossius[16] have shown that, if bilirubin be injected intravenously, a very marked increase in the excretion of bile pigment results. There can therefore be no doubt that when bile pigment is formed elsewhere, it may be excreted by the liver.

A large number of researches has been instituted to decide the question as to whether the liver is a seat of the formation of bile pigment as well as of its excretion. The following observations are the most important results of these researches.

Fleischl[13] (1873) proved that after ligature of the common bile duct in mammals, the bile pigments and the salts of the bile acids could be detected first in the lymph, later on in the blood and urine. If the common thoracic duct be ligatured at the same time the appearance of bile pigments in the blood and urine is much delayed.

In 1885 Stern[2] showed that ligature of the bile duct in the bird resulted in the rapid production of universal icterus. The presence of bile pigment in

in the blood serum and urine was also demonstrated. The urine was obtained free from faeces by previously ligaturing the alimentary canal above the ureters. On the other hand if all the vessels passing to and from the liver be ligatured at the same time as the duct, no bile pigments or salts of bile acids could be detected in the lymph, blood or urine.

Minkowski and Naunyn[4] completed the proof by excision of the liver of geese. After this operation the birds lived only ten to twenty hours. No bile pigments or salts of bile acids could be detected in the lymph, blood or urine even after the administration of arseniuretted hydrogen, which in normal birds is found to produce so large an increase in the quantity of bile formed that the rapidity of excretion falls behind that of formation with the result that part of the bile is absorbed and universal icterus is produced.

It has already been seen that blood pigment is to be looked upon as the mother substance of haematoidin which is almost universally regarded as identical with bilirubin; but the genetic relationship of bile pigment with blood pigment may be proved even apart from the previously stated facts. The injection of oxyhaemoglobin into the blood stream

stream and also all chemical agents e.g., bile salts, water, glycerin, arseniuretted hydrogen, totuylene-diamine, pyrogallic acid etc., which on entering the blood stream set free oxyhaemoglobin from the blood corpuscles, produce an increase in the quantity of bile pigment excreted. If the quantity of blood pigment dissolved in the plasma be small, the whole is as a rule converted in the first instance into bilirubin. If larger quantities of blood pigment be present in the plasma part of the oxyhaemoglobin escapes the action of the liver, and is excreted in the urine, partly as such, partly as methaemoglobin and haematin. The formation of bilirubin from blood pigment involves first the decomposition of the latter into globin and haematin and secondly the separation of iron from the haematin molecule in accordance with the equation previously given. It is still uncertain whether both stages of the decomposition are carried out by the liver alone. Pugliese [26] has brought forward some evidence in favour of the view that the spleen plays a part in the decomposition. He states that after removal of the spleen the excretion of bile falls to about half its original amount, while the specific gravity and total amount of solids of the bile remain unaltered.

unaltered.

Only a small proportion of the iron resulting from the decomposition of the blood pigment is excreted. About .007 gms. of iron per litre is excreted in the urine. Kunkel(9) found that for each 100 parts of bilirubin formed by the liver only 1.4 to 1.5 parts of iron are excreted in the bile; while 100 parts of haematin would yield nine parts of iron. Minkowski and Naunyn (loc.cit.) have also shown that the quantity of iron in the bile is unaltered after the administration of arseniuretted hydrogen; although the quantity of bile pigment excreted is much increased. The larger proportion of the iron appears to be retained in the liver in loose chemical combination with albuminous substances. (18,19,20,21) Nucleoproteids are also found containing iron in more stable combination. (10)

With regard to the farther history of the products of decomposition of blood pigment within the body, part of the bile pigment excreted appears to be converted within the alimentary canal into stercobilin. The results of the work of Garrod, Hopkins (22,24) and others prove that this pigment has the same percentage composition and chemical characters as the urobilin of the urine. The stercobilin is absorbed and excreted partly as urobilin, partly

partly probably also as urochrome. The latter appears to be the chief normal urinary pigment. The fact that it is converted into a body resembling urobilin by the action of aldehyde which may be regarded as acting as a feeble reducing agent, seems to indicate that it is a product of the oxidation of urobilin. A colourless chromogen of urobilin--urobilinogen--is also found.

Saillet[23] holds that in freshly passed urine the chromogen alone is present. Under the influence of light and oxygen the latter is converted into urobilin. Dilute mineral acids, ammonia, and oxidising agents produce the same transformation.

Haematoporphyrin, frequently, uroerythrin, and possibly urorosein, are the only other preformed pigments whose presence in normal urine has been definitely proved. Haematoporphyrin is also in part excreted as a chromogen. It is still doubtful whether uroerythrin and urorosein are products of the Katabolism of blood pigment. The latter is regarded by Hammarsten as being possibly derived from indoxyl or skatoxyl.[27]

The consideration of the Katabolism of blood pigment appears to indicate that the pigments of bile and urine with the exception of urochrome are

are formed under conditions which favour reduction rather than oxidation. It has already been seen that bilirubin is formed in regions where the oxygen tension is low e.g., into cysts into which blood has been extravasated and remained cut off from the general circulation for long periods.
Further the bile is secreted mainly if not entirely from venous blood. A consideration of the chemical methods employed for the decomposition of haematin will also be found to prove that the iron is most readily detached from the haematin molecule in the presence of reducing agents. These, along with other facts, led Hoppe Seyler to express the opinion "that the formation of bilirubin from haemoglobin occurs under the influence of an acid, and of water, in the absence of oxygen."

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Chemical Methods used for the Reduction of Haematin.

1. Reduction of Haematin in Alkaline Solution.

Stokes in 1864 noted that, on the reduction of haematin in alkaline solution by means of ferrous hydrate dissolved in an alkaline tartrate, a body was obtained having spectroscopic characteristics quite distinct from those of haematin. The solution of haematin employed by Stokes being obtained by the decomposition of oxyhaemoglobin contained proteids. It showed after reduction two well marked absorption bands, one a dark well marked band ~~and~~ extending from D25E - D63E, and a fainter band B which commences a little to the red side of E and reaches a short distance beyond b. On shaking up the solution of this body with the air, it is oxidised again to alkaline haematin, the bright red colour of the reduced pigment passing into the greenish red of alkaline haematin. Stokes therefore named the pigment reduced haematin.

Later Hoppe Seyler(1) showed that a body having absorption bands identical with those observed by Stokes in solutions of "reduced haematin" may be obtained by the decomposition of reduced haemoglobin by means of alkalies. This body he termed haemochromogen. On the other hand, the reduction in

in alkaline solution of pure haematin prepared from its hydrochloride--haemin--does not invariably lead to the formation of a body possessing the spectroscopic characters of haemochromogen. Hoppe Seyler (loc. cit.) found that for the formation of a pigment having the spectroscopic characters of haemochromogen by the action of reducing agents on haematin, the presence of certain impurities was necessary. Amongst these impurities he mentions proteids, ammonia and asparagine. Gamgee confirmed this observation. He made the further observation that although oxidised haemochromogen is identical in appearance and visible spectroscopic characters with alkaline haematin, they are probably distinct bodies.

"A strong proof that oxidised haemochromogen is not identical with haematin is derived from my own observations on the absorption of the extreme violet and ultra violet. Whilst haematin possesses even in solutions of great dilution the power of absorbing the whole of the ultra violet, the violet, and even the blue rays of the solar spectrum, oxidised haemochromogen is, in solution of much greater concentration, remarkably transparent for the ultra violet."

In 1890 Bertin-Sans and Moitessier[2] investigated this subject more fully and found that if such re-

* Text book of Physiology ed. by Schäfer vol I p. 251

reducing agents as potassium sulphide, ferrous tartrate or sodium hydrosulphite be added to a solution of alkaline haematin the typical spectrum of haemochromogen does not appear. In order to obtain the absorption bands of a haemochromogen it is necessary to add to the solution ammonia, an amine (e.g. ethylamine or aniline) an amido acid (e.g. glycine or taurine) or a trace of proteid. Urea is without influence on the spectral appearances.

The most complete investigation of the properties of haemochromogen was carried out by Zeynek, who first succeeded in separating and analysing this body. The apparatus employed for this purpose is somewhat complicated, essentially the method consisted in the reduction of an ammoniacal solution of haematin by means of hydrazine hydrate. The brownish green solution of alkaline haematin on being slightly warmed with hydrazine hydrate yielded a cherry red solution of haemochromogen, the nitrogen set free by the decomposition of the hydrazine hydrate being simultaneously evolved. For separating the haemochromogen from solution Zeynek took advantage of the fact that haemochromogen is insoluble in a mixture of alcohol and ether. The reduction, precipitation, and drying of the precipitated haemochromogen were carried out in a current of dry hydrogen.

hydrogen. Zeynek ultimately succeeded in considerably simplifying the method of preparation. In his later work he reduced haematin partly dissolved, partly suspended in ammoniacal alcohol, instead of reducing haematin in watery solution. He thus avoided the labour of getting rid of the water used for solution. Analyses of the haemochromogen thus prepared gave higher values for the nitrogen than were anticipated. It was found that the high percentage of nitrogen was due to the fact that the haemochromogen was an ammonium compound. He found the following formulae to agree best with the percentage composition <u>either</u>

$$C_{32} H_{35} Fe N_5 O_3 \quad \underline{or} \quad C_{64} H_{70} Fe N_{10} O_7,$$

Assuming the correctness of Nencki's formula for haematin namely $C_{32} H_{32} Fe N_4 O_4$, the ammonium salt of haematin would have the formula $C_{32} H_{35} Fe N_5 O_4$, and the relationship between the ammonium compound of haematin and that of haemochromogen might be symbolised by either of the two following equations.

$$\underset{\text{(ammonium compound of haemochromogen)}}{C_{32} H_{35} Fe N_5 O_3} + \underline{O} = \underset{\text{(ammonium compound of haematin)}}{C_{32} H_{35} Fe N_5 O_4}$$

$$\underline{or}\ \underset{\text{(ammonium compound of haemochromogen)}}{C_{64} H_{70} Fe N_{10} O_7} + \underline{O} = 2\underset{\text{(ammonium compound of haematin)}}{C_{32} H_{35} Fe N_5 O_4}$$

haematin. The results of the analyses agreed almost equally well with either of the two above formulae for the ammonium compound of haemochromogen. From this ammoniacal compound Zeynek tried to obtain free haemochromogen by neutralisation with dilute acetic acid; but as yet has not been successful. No separation of iron from the haemochromogen takes place on adding the acetic acid. This was proved by the fact that on rendering the solution alkaline the spectrum of haemochromogen reappeared. It is noteworthy that by the reduction of haematin in ammoniacal solution by hydrazine hydrate haemochromogen is the only reduction product obtained so that it appears that the action of hydrazine hydrate on an ammoniacal solution of haematin reaches its termination with the formation of haemochromogen.

The first to fully study the later products of the reduction of haematin in alkaline solution was Hoppe Seyler.[11] He used as his reducing agent zinc dust added to a solution of haematin in caustic soda. The first perceptible spectroscopic change is that the ill defined band of alkali haematin lying in the orange and yellow becomes more sharply limited, displaced towards the red end of the spectrum and ultimately replaced by a narrow sharply limited band lying nearly midway between C and D. Simultaneously

Simultaneously a band appears immediately to the violet side of D, and a very broad diffuse band starting from E and reaching nearly to a region midway between E and F. On further heating the alkaline haematin solution with zinc dust on the water bath these spectroscopic appearances remain unaltered for a considerable time. The iron at this stage was found to have been separated from the haematin molecule. On acidifying the solution a brownish red precipitate and purple red solution were obtained. Hoppe Seyler unfortunately did not examine the acid solution spectroscopically.

After still more prolonged action of the zinc on the original solution an absorption band appeared between D and E nearer to the latter line and finally a fifth band lying between C and D close to the former line. On continued boiling of this solution no other change was noted in the spectroscopic appearances, and it was concluded that the chemical action here reached its termination. Acidification with hydrochloric acid produced a precipitate which was washed, dried to constant weight and analysed. The elementary analyses of the substance so obtained gave such different results that Hoppe Seyler concluded that the substance so obtained was a mixture.

Several grammes of the product of reduction of haematin in alkaline solution were distilled with zinc dust. The vapours formed gave a distinct pyrrol reaction. In addition colourless oily drops which afterwards became yellow or brown distilled over. From a solution of the substance in hydrochloric acid caustic soda precipitated a yellow coloured pigment while the fluid in which the precipitate was suspended acquired a purple tint. The spectroscopic characters of these pigments were not described (see p. 536).

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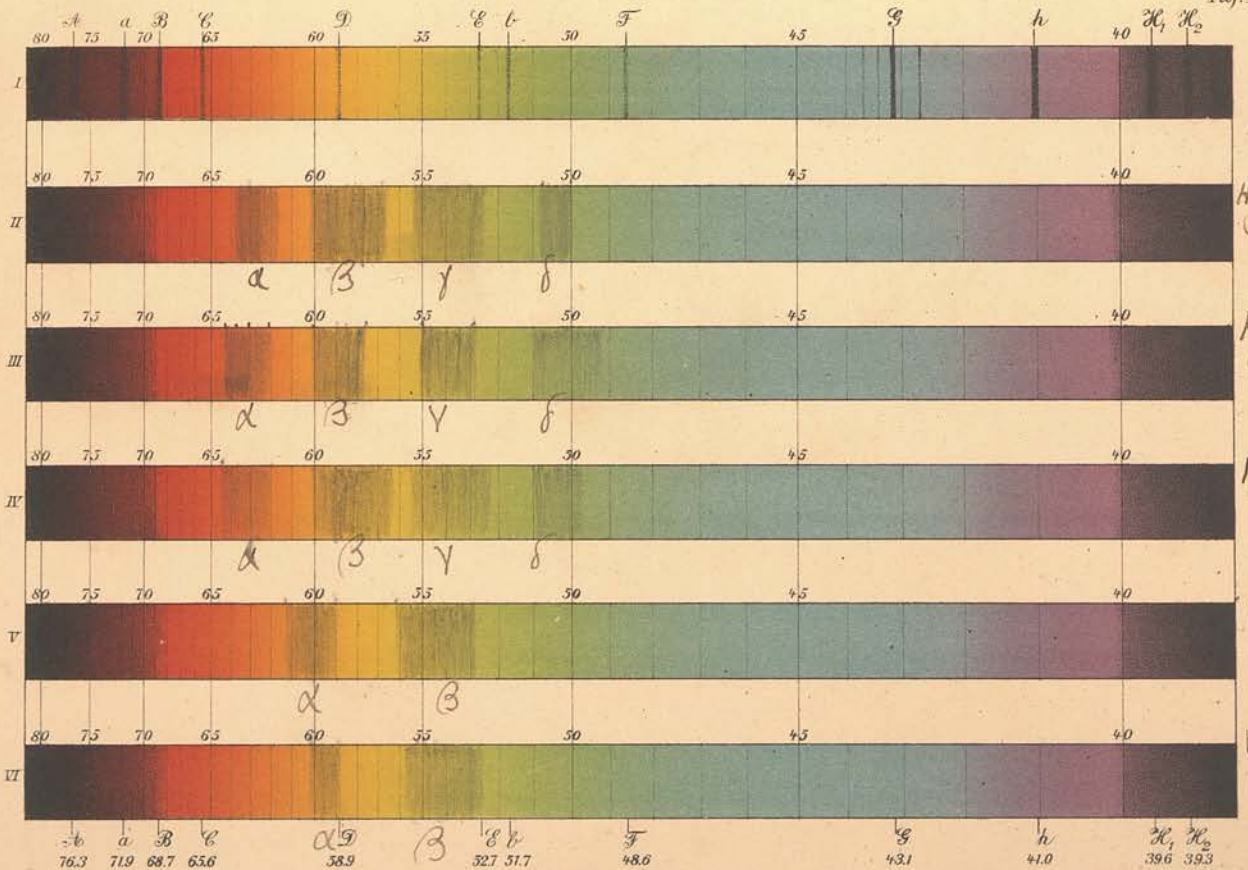

Haematoporphyroïdin (alkaline solution) Fig 1

Haematoporphyrin (alkaline solution Fig 2.

Haematoporphyroïdin (fourthy acid solution) Fig 3.

Haematoporphyrin (acid solution) Fig 4

Haematoporphyroïdin (strongly acid solution. Fig 5

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Le Nobel(6) used Zinc and Tin in alkaline solution as reducing agents and carried out the reduction at room temperature. He first noted that fluid became red and showed the bands of reduced haematin. Presuming that a fixed alkali was used, the appearance of these bands must have been due to some impurity adhering to the haematin employed. A little later iron is split off from the haematin and haemotoporphyrin is formed (spectrum figure 2) The latter pigment was precipitated by neutralisation and recognised in all its properties. As the reduction of the original solution is continued the α band becomes fainter, B and γ more intense (spect./ fig.3) The body was precipitated by neutralisation with acid and recognised as identical with a body also obtained by reduction of haematin in acid solution. To this pigment Le Nobel gave the name haematoporphyroidin. As the reduction of the original solution is continued the spectrum alters to that shown in figure of the spectral chart. Le Nobel recognised this, the final product, as identical with a body also obtained by the reduction of haematin in acid solution which he termed isohaematoporphyrin. Nencki showed later that in strongly acid solution the so-called isohaematoporphyrin showed the spectrum of haematoporphyrin in acid solution.

Products of the reduction of haematin in ammoniacal solution
(Macnamara)

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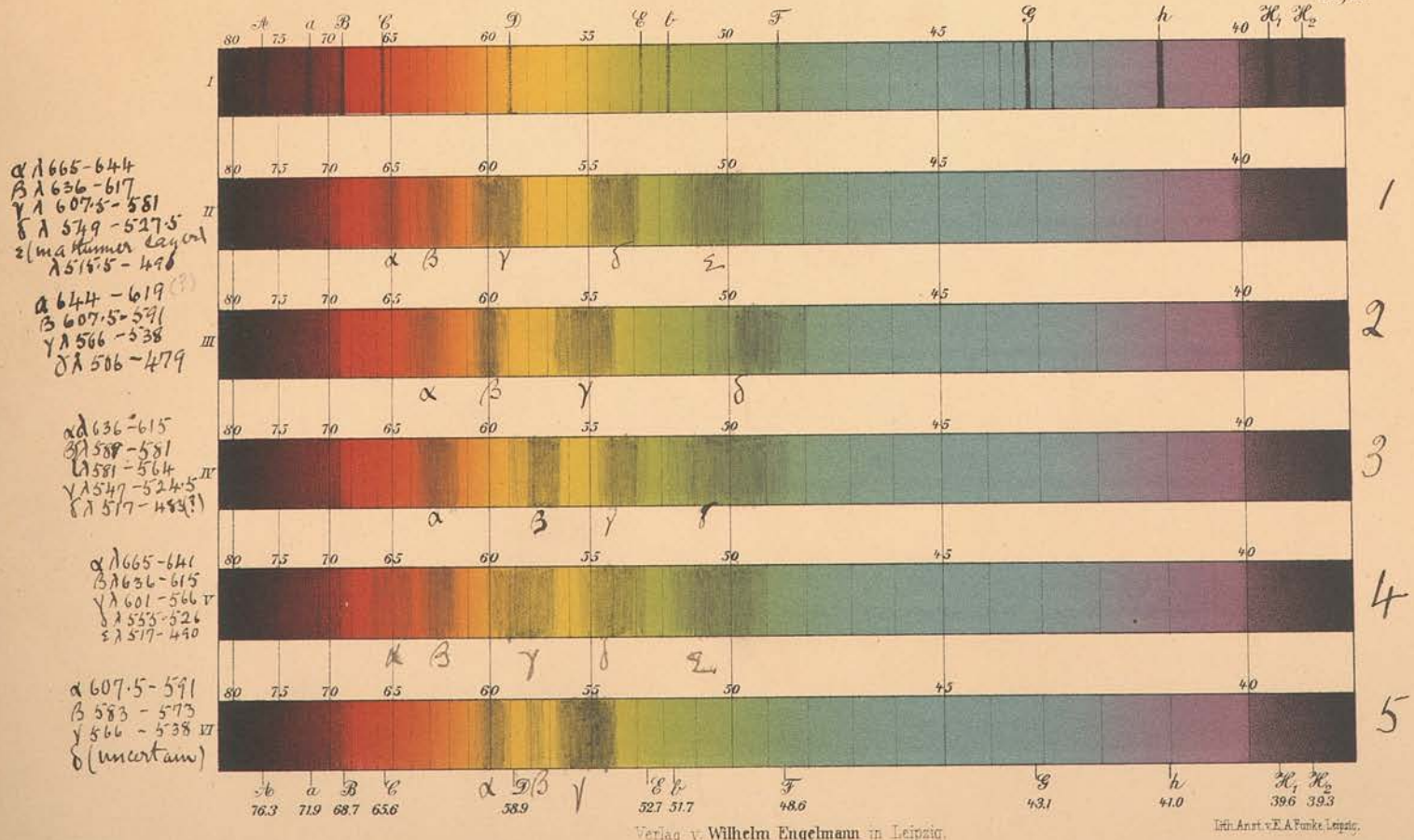

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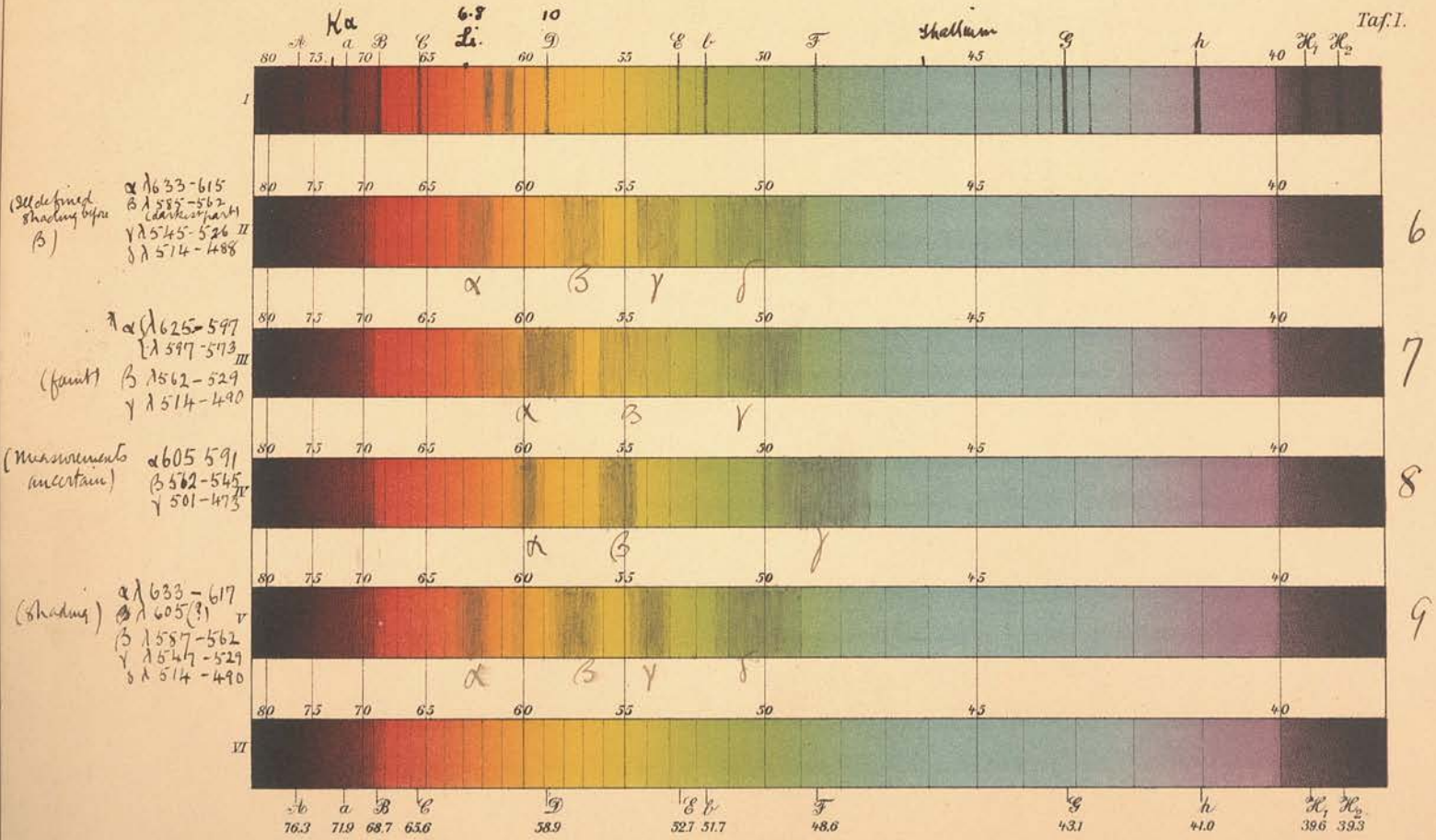
solution.

MacMunn(3) was the next observer who worked at the products of the reduction of haematin in alkaline solution. Haematin was dissolved in rectified spirit containing a little aqueous caustic soda and reduced by means of sodium amalgam. The fluid was gently heated on the water bath for 35 minutes and then showed the spectrum given in figure 1 of the spectral chart[7]. On acidification the fluid became a bright red colour and gave spectrum 2. "The solution was then agitated with chloroform (after dilution with water)." The chloroform took up all the pigment, became a purple red and yielded on evaporation a violet brown residue the solution of which in rectified spirit was of a purplish tint and gave spectrum 3. The substance or mixture of substances having the above spectroscopic appearances was called "the first reduction product."

On further reduction a solution was obtained showing spectrum 4 "second reduction product." A portion was acidified with sulphuric acid, filtered and then showed spectrum 5. The residue from a chloroform solution of the pigment was dissolved in rectified spirit rendered alkaline by ammonia and gave spectrum 6. No alteration in the bands or in the appearance of the solution was produced by the



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the addition of zinc chloride.

"After half an hour's further action the fluid showed a new spectrum, and on standing exposed to the air it assumed an orange colour and gave spectrum 7. On distinctly acidifying the solution with sulphuric acid, spectrum 8 was obtained." An ammoniacal solution in rectified spirit of the residue from a solution of the pigment in chloroform gave spectrum 9. On addition of zinc chloride to this solution a faint green fluorescence was perceptible and the 4th band on spectroscopic examination was found to be intensified.

2. Reduction of Haematin in Acid Solution by means of Metals.

(1 and 2)
Hoppe Seyler investigated the action of tin and hydrochloric acid upon haematin in alcoholic solution. The results, when excess of haematin was used, differed from those obtained when a more dilute solution of haematin was acted upon.

If a concentrated alcoholic solution of haematin containing some of the pigment in suspension be heated on the water bath with tin, zinc or copper and hydrochloric acid, the fluid soon acquires a beautiful purple red tint, and a resinous dark violet precipitate separates out. This precipitate is only slightly soluble in acid alcohol, insoluble in ether and does not contain iron; but even after prolonged heating with strong hydrochloric acid cannot be obtained free from tin. On heating the dried precipitate it yielded a blue non-crystalline sublimate, and oily drops which became red on exposure to the air. The fluid, from which this precipitate had separated out, showed, on spectroscopic examination, two dark absorption bands lying between D and E, one of which lies nearer to D but separated from it by a distinct interval while the other borders directly on E.

Hoppe Seyler next carried out the same experiment

experiment using in this case a more dilute solution of haematin and heating the solution on the water bath. The fluid soon acquired a purple tint; but no resinous body separated out. On spectroscopic examination an absorption band was observed lying between D and E, and another immediately to the red side of D. Later a third very broad ill defined band appeared lying between B and F. The spectroscopic appearances seem to correspond to those of a mixture of acid haematoporphyrin plus some other pigment spectroscopically resembling urobilin. On continuing the reduction the band between D and E moves nearer to E gradually becomes fainter and ultimately disappears. The mixture is finally almost yellow in colour and shows on spectroscopic examination a well defined narrow band close to F, the two other bands having entirely disappeared. Hoppe Seyler next separated the pigment by the following somewhat complicated method. The fluid was concentrated to small bulk, then poured into excess of boiling water. A brownish precipitate formed which was filtered off. From the filtrate more of the pigment was obtained by partial neutralisation with a dilute solution of caustic soda. The pigment was then dissolved in alcohol. By precipitation with lead acetate, decomposition of the precipitate with

with oxalic acid and solution in alcohol further portions of the pigment were obtained. The alcoholic solution was freed from oxalic acid and filtered. A brownish purple red residue is left after evaporation of the alcohol. The residue is readily soluble in alcohol and yields with it a purple red solution. It could not be obtained in crystalline form in slow evaporation of its alcoholic solution over sulphuric acid. The spectroscopic characters were unfortunately not described. The results of elementary analysis of the pigment indicated that the changes undergone by the haematin essentially consisted in a separation of iron and an addition of hydrogen atoms, no oxygen being removed from the haematin molecule.

In 1884 Nencki and Sieber[7, 8,] undertook the study of the action of metals plus mineral acids upon haemin in alcoholic solution. 5 gms. of haemin were mixed with one litre of 93 per cent alcohol to which were added 100 ccm. of pure hydrochloric acid (sp. gr. 1.12) and some tin foil. The mixture was then heated for five hours on the water bath in a flask provided with a reflux condenser. The fluid had then acquired a yellowish colour and showed on spectroscopic examination one band corresponding in position to that of Urobilin. The solution was next

next filtered and concentrated to a third of its original bulk. After the concentrated solution had remained exposed to the air for five or ten hours, a brownish red pigment separated out in the form of granules showing no trace of crystalline structure. The pigment so obtained is insoluble in ammonia and fixed alkalies, very slightly soluble in dilute hydrochloric acid, but readily soluble in alcohol. The alcoholic solution is dark red in colour and shows on spectroscopic examination three absorption bands, two narrow bands lying between D and E, and a broad band between b and F corresponding in position to that of Urobilin. For further purification the pigment was dissolved again in alcohol, and the filtrate was then boiled with ammoniacal alcohol in order to free it from Tin and Iron. Notwithstanding repeated purification by this means the pigment yields stannic oxide on incineration.

Elementary analyses of the pigment residue obtained from its alcoholic solution by evaporating off the alcohol indicated that it was a hexahydrohaematoporphyrine. According to Nencki and Sieber, its formation may be symbolised by the following equation:- $C_{32}H_{30}N_4FeO_3Cl + 2H_2O + HCl + H_2 =$

(haemin)

$C_{32}H_{38}N_4O_5 + FeCl_2$

(hexahydrohaematoporphyrine)

(hexahydrohaematoporphyrine)

In addition to hexahydrohaematoporphyrine there was formed a small quantity of another pigment bearing a very close resemblance to Urobilin. The latter pigment is soluble in alkalies yielding solutions which are brownish red when concentrated, amber yellow when dilute. On faintly acidifying the alkaline solution the pigment is precipitated in the form of reddish brown flocculi. Saturated acid solutions of the pigment are yellowish red, and become on dilution rose red. On spectroscopic examination they show one band corresponding in position to the band of Urobilin. This pigment may be obtained in larger amount by adding more acid and Tin than was used in the method of preparation already described and by heating the fluid for a longer period. Nencki and Sieber suggest that the formation of this colouring matter may be expressed by the following equation:-

$$C_{32}H_{30}N_4Fe O_3 + 4H_2O + 2HCl = C_{32}H_{40}N_4O_7 + FeCl_2$$

(Maly's hydrobilirubin)

On heating the alcoholic haemin solution for a still longer period with tin and hydrochloric acid, the fluid becomes almost colourless and has an odour resembling that of pyridine. Further, on rendering the solution alkaline and subjecting it to distilla-

Products of reduction of haematin
in acid solution (Maximum

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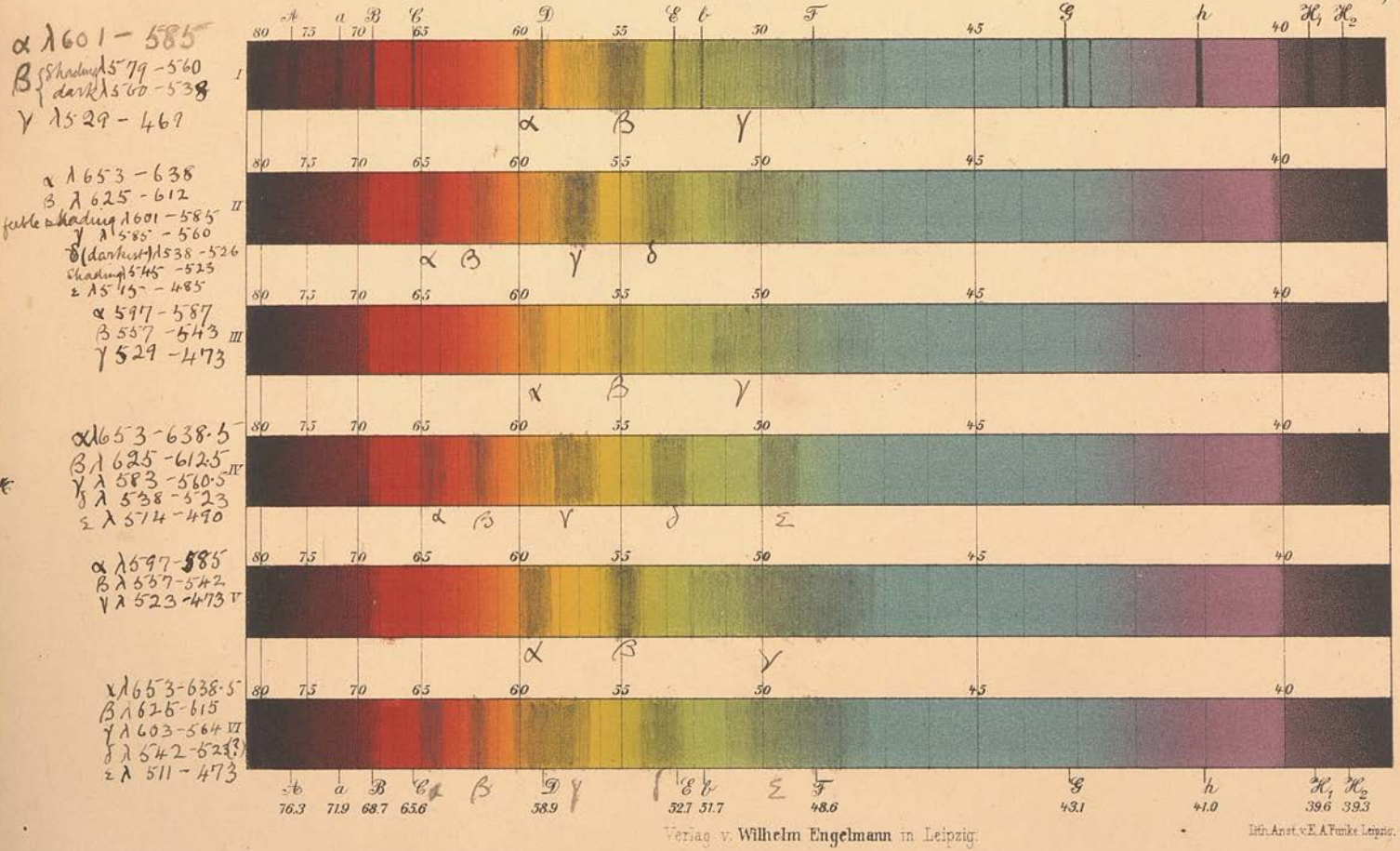

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distillation, a volatile body distils over which is soluble in water and gives an intense red colour to a pine wood strip dipped in hydrochloric acid. The latter reaction is strong evidence that the volatile substance is a pyrrol derivative.

I shall next give a brief account of MacMunn's work on this subject. In the following description I have mainly followed the exposition given in his last paper (Journal of Physiology, Vol. 10, pages 87-91). Haematin was dissolved in rectified spirit containing sulphuric acid and the solution was heated with zinc on the water bath. "In about an hour the band in the red of acid haematin had gone and the fluid had become red. The solution was then filtered. It had a deep purple red tint, and showed spect. 1." This pigment, termed by MacMunn "the first reduction product," was then dissolved in chloroform and the pigment residue left after evaporation of the chloroform was dissolved in rectified spirit containing ammonia. On spectroscopic examination the solution in ammoniacal rectified spirit showed spectrum 2.

"Second reduction product."

"The solution from which the above pigment had been removed was again heated with zinc and sulphuric acid for about an hour. The solution had then be-

Products of the reduction of haematin in acid solution / Maximum

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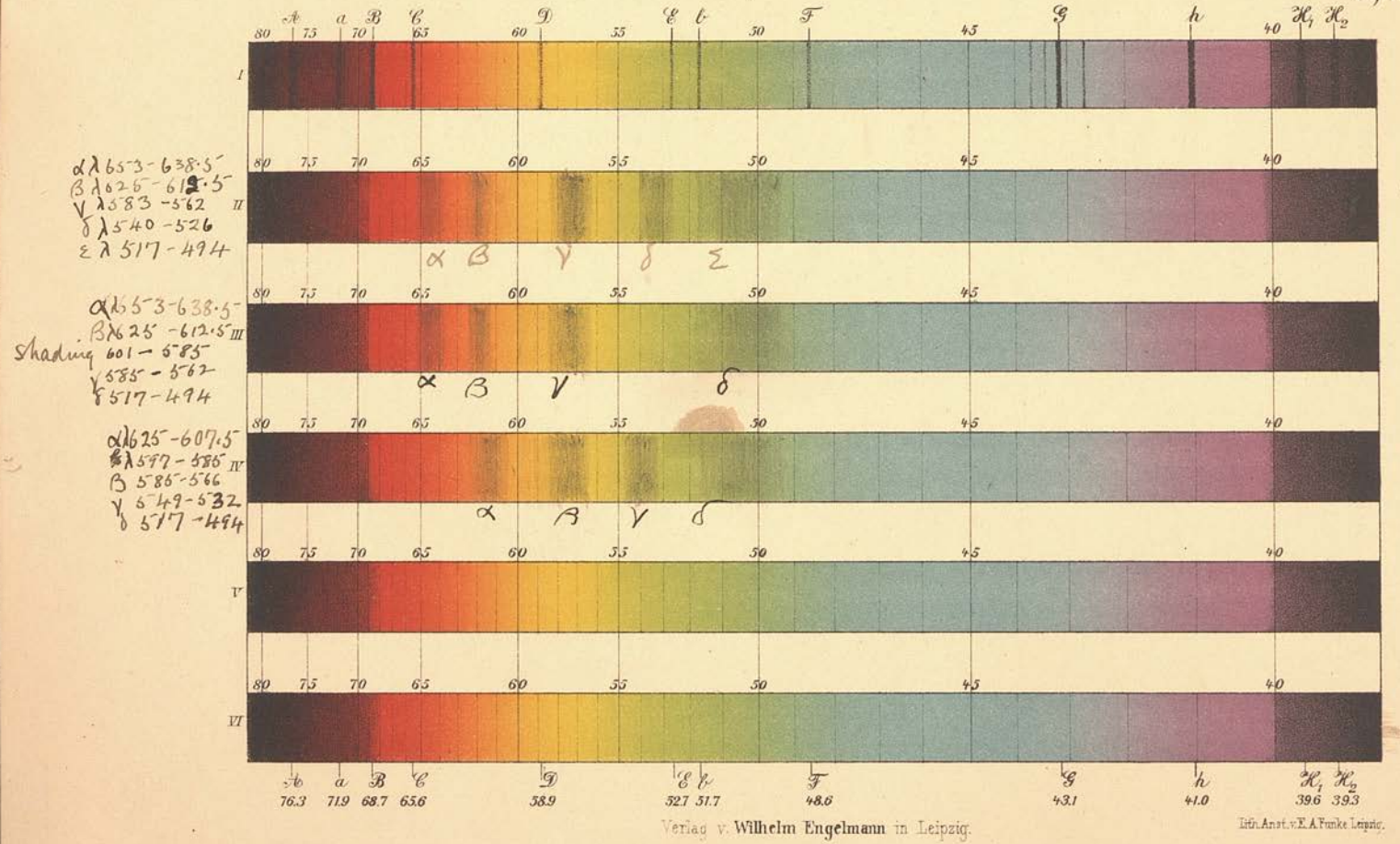

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become red brown, and was lighter in colour. Now the band at D and that in the green, were lighter than before, that at F being much darker (spect. 3). A chloroform extract of this solution left on evaporation a sepia brown amorphous residue, which was dissolved in rectified spirit." A solution in rectified spirit containing ammonia showed spect. 4. With zinc chloride it gave a faint green fluorescence.

"Third reduction product."

"The solution from which the last had been removed was again heated with zinc and sulphuric acid on the water bath for about an hour." The fluid had then become yellow in colour. "It showed, on spectroscopic examination, a feeble shading at D and a dark band at the blue end of the green. After standing exposed to the air the fluid became darker until it became of an orange colour. It then gave spect. 5." A solution in chloroform yielded on evaporation a brown residue which was dissolved in rectified spirit "The rectified spirit solution was deep red in concentrated solution and gave spect. 6. On addition of ammonia the spectrum 7 appeared." To this solution MacMunn gave the name alkaline urohaematoporphyrine. On addition of zinc chloride and ammonia the solution showed a green fluorescence and gave

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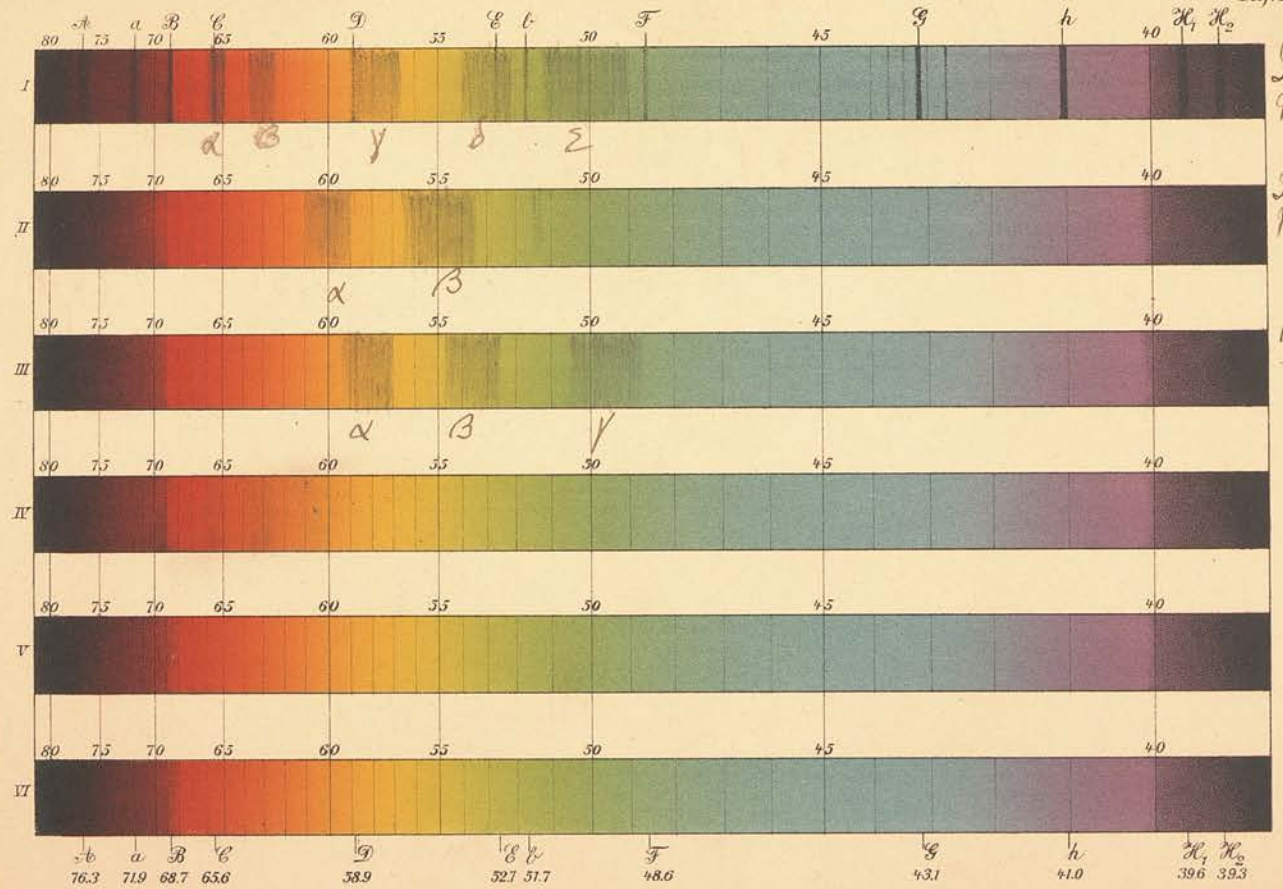

Isohaematoporphyrin (alcoholic solution) — Fig I

Isohaematoporphyrin (strongly acid solution) — Fig 2

Hexahydrohaematoporphyrin (alcoholic solution) — Fig 3.

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gave spectrum 8.

Le Nobel's Observations.

(loc. cit.)

Le Nobel dissolved haematin in alcohol containing sulphuric acid and reduced with zinc or tin at room temperature. The first change noted was that the band of acid haematin in the red gradually faded, and concurrently with the gradual disappearance of the band of acid haematin in the red, a dark absorption band appeared in the green between D and E. A narrow sharply limited dark band also appeared close to the red side of D (spect. 4 ch 1). A small portion of the fluid was then removed from the flask in which the reduction was carried out, and rendered alkaline. The spectrum of the alkaline solution is shown in fig. 2 of the spectral chart I. Another portion on neutralisation yielded a precipitate. The spectroscopic characters of this pigment are evidently those of haematoporphyrin. The first product of the reducing action is therefore haematoporphyrin. On more prolonged action of the reducing agent the fluid became of a reddish brown colour but no distinct change in its spectroscopic characters was apparent. On neutralising a portion of the fluid a reddish brown precipitate was obtained. This precipitate was insoluble in neutral alcohol, readily soluble in faintly acid alcohol and in dilute alka-

alkalies. The latter solutions showed the same bands on spectroscopic examination (fig. 1 of the spectral chart). In strongly acid alcohol the pigment yielded a solution which showed on spectroscopic examination two bands almost identical in position and character with those of haematoporphyrin in acid solution. On account of its close resemblance to haematoporphyrin Le Nobel named this pigment haematoporphyroidin. A little later the colour of the solution had become somewhat browner although no change was noted in its spectroscopic characters. A portion of the fluid was again taken and on neutralisation yielded a precipitate which on solution in neutral (e.g., alcohol or chloroform), alkaline, or faintly acid media gave a spectrum with five absorption bands (fig. 1, ch. 4). On strongly acidifying the alcoholic solution with sulphuric acid the five bands were replaced by two bands identical in position and character with the bands of acid haematoporphyrin. Le Nobel gave to the pigment having the above mentioned characters the name isohaematoporphyrin.

Le Nobel next heated isohaematoporphyrin on the water bath with zinc or tin and hydrochloric acid. From time to time a small portion of the solution was taken and neutralised with dilute caustic potash.

potash. At the commencement of the action the precipitate which forms on neutralisation is a mixture of isohaematoporphyrin with a body which apparently closely resembles Urobilin.

Finally the latter substance which Le Nobel named Urobilinoidin alone remained. From these appearances Le Nobel concluded that no body intermediate between isohaematoporphyrin and urobilinoidin was formed during the reduction. At the stage at which both these pigments were present they could be partially separated from one another by diluting the fluid with water and shaking with chloroform. Urobilinoidin first passed into the chloroform. If the chloroform solution of urobilinoidin be evaporated down to dryness the pigment residue is found to give a green fluorescence with ammonia and zinc chloride. Its solution showed a band near F corresponding to that of urobilin. If however a solution of urobilinoidin in chloroform which at first showed only one band at F were left exposed to the air for several hours, the solution was found to have acquired a red brown colour and showed on spectroscopic examination five bands apparently identical in position and character with those of isohaematoporphyrin. Le Nobel therefore inferred that by the reduction of haematin it was impossible to obtain a

a pigment which would more or less permanently show only a single band at F and drew the conclusion that there was no relation between urobilin or hydrobilirubin and the pigment obtained by the reduction of haematin. He regarded the urohaematin or urohaematoporphyrin of MacMunn as consisting of a mixture of isohaematoporphyrin with urobilinoidin. He also found that from the hexahydrohaematoporphyrin of Nencki and Sieber urobilinoidin might be extracted by means of chloroform, and therefore concluded that it was also a mixture.

Nencki and Sieber's later Observations.

Nencki and Sieber(8) in their later work directed special attention to an attempt to isolate the body resembling urobilin which results from the prolonged reduction of haemin or haematin. 20 gms. of haemin were mixed with 400 gms. of 93 per cent alcohol previously saturated with hydrochloric acid. To this was added iron in the form of fine wire. The mixture was then heated on the water bath in a flask with reflux condenser. The haemin passed gradually into solution, and was in the first instance converted into haematoporphyrin identical with that prepared by the action of glacial acetic acid saturated with hydrobromic acid. The solution was boiled until it had acquired an orange yellow colour. Heat-

Heating for six hours was found necessary to produce this transformation. The precipitate which formed on pouring the filtered solution into large excess of water, was filtered off, and then boiled with alcoholic ammonium sulphide in order to remove the iron. The alcoholic solution thus obtained was freed from sulphide of iron by filtration, concentrated to a volume of about 400 ccm. and poured into excess of water. A body resembling urobilin passed into the aqueous solution; while the precipitate, which formed, yielded on solution in alcohol only a faint fluorescence with ammonia and zinc chloride. In order to isolate the pigment which dissolved in water, its aqueous solution was concentrated to half its original volume and extracted with ether. The ethereal extract left on evaporation only a small amount of residue. This pigment residue was found to be soluble in water, dilute alkalies and dilute acids. In all these solvents the pigment showed on spectroscopic examination a well marked band corresponding in position to that of urobilin. Its ammoniacal solution also gave a green fluorescence with zinc chloride. An ammoniacal solution of the pigment on remaining for a few days exposed to the air altered in colour becoming brownish and no longer showed a green fluorescence on addition of zinc

zinc chloride. Further investigation of this pigment was rendered impossible owing to the smallness of the quantity obtained.

Observations made by Eichholz. (17)

"Haematin prepared by either Kűhne's or MacMunn's methods was reduced on the water bath with either tin or zinc and hydrochloric acid, tin being preferable to zinc." Products similar to those obtained by MacMunn and Le Nobel were observed during the earlier stages of the reduction. Eichholz, however, carried the reduction to a later stage than either MacMunn or Le Nobel.

"The reduction was continued with replacement of acid and tin when necessary until the solution became almost colourless and on filtering acquired a yellow colour; but did not revert to the urohaematoporphyrin stage. This yellow solution was left to stand for 18 hours and did not change. It was then extracted with chloroform and evaporated to dryness, yielding a light brown amorphous residue. This residue was soluble in absolute alcohol and cold water. The alcoholic solution of the residue closely resembled urobilin in its band at F, its slight green fluorescence and light brown colour. With hydrochloric acid the band remained, resembling that of urobilin. The solution showed a slight green

green fluorescence. With ammonia the colour of the solution became fainter, and the fluorescence disappeared. Zinc chloride and ammonia gave a pale yellow solution with green fluorescence, with a single band similar to the band of urobilin (under) the same conditions. Zinc chloride in neutral solution gave a pink colouration and a light green fluorescence. Caustic soda shifted the band toward the red. Sodium amalgam caused the band to disappear from the solution. Addition of acid, however, immediately restored it."

"The solution of this artificial pigment differs from normal urobilin in the slight fluorescence of its acid solution and in the marked tendency on long standing to decompose so as to show a band in the region of D)) 570 -)) 598."

Review and interpretation of the Results obtained by the Reduction of Haematin in Alkaline and Acid Solution.

The difficulty of arriving at definite conclusions with regard to the nature of the pigments formed by the reduction of haematin is largely due to the fact that the spectroscopic characters of a solution containing haematoporphyrin vary much according to the mode of chemical combination in which the pigment occurs. Neglect of due attention to this fact has led to much confusion and has induced some observers to regard certain of the compounds of haematoporphyrin as distinct chemical substances. Haematoporphyrin may be found in solution either in the free form or in combination with other substances. It plays the double part of acid or base according to the chemical conditions under which it is placed. In the presence of bases it plays the part of an acid forming salts of which the following have been prepared, (1) Sodium salt ($C_{16}H_{17}Na\,N_2O_3 + H_2O$); (2) Silver salt ($C_{16}H_{17}Ag\,N_2\,O_3 + H_2\,O$) and (3) Zinc salt ($C_{16}H_{16}Zn\,N_2O_3 + H_2O$). Barium and calcium salts have also been obtained, but, so far as I am aware, not analysed. The formulae of the silver and sodium salts seem to indicate that haematoporphyrin

haematoporphyrin is a monobasic acid, while that of the zinc salt suggests that it is a dibasic acid. As will be seen later the zinc salt shows spectroscopic characters differing from those of the sodium salt. All the metallic compounds of haematoporphyrin, with the exception of those with the alkaline metals and ammonia are insoluble in water. In the presence of mineral acids haematoporphyrin plays the part of a base forming salts, of the most thoroughly examined of which is the hydrochloride ($C_{16}H_{18}N_2O_3$ Hcl). It is doubtful whether haematoporphyrin forms salts with organic acids. A solution of haematoporphyrin in glacial acetic acid as will be seen later shows a totally different spectrum from its solution in a dilute mineral acid. The latter fact would seem to indicate a difference in the chemical nature of the two compounds.

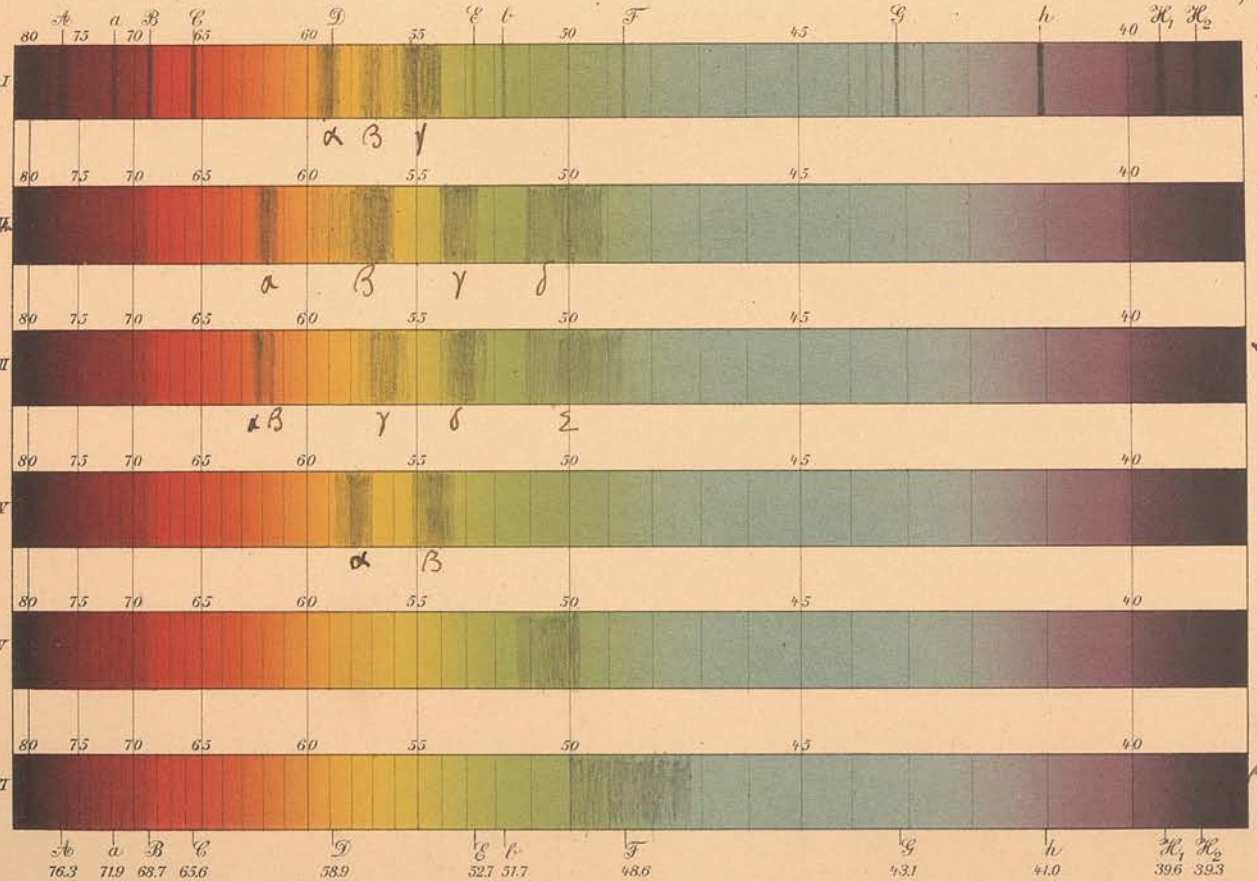
Nencki and Sieber have shown that haematoporphyrin owes its acid character to the presence of two hydroxyl groups. If the hydrogen atoms of these hydroxyl groups be replaced by alkyl radicles the ethereal compounds of haematoporphyrin so obtained are found to be insoluble in aqueous alkalies, as a result of the fact that a hydrogen atom replaceable by metals is no longer available. The alkyl derivatives of haematoporphyrin are therefore incapable of

of playing the part of acids although they still react towards mineral acids as bases. The dimethyl ($C_{16}H_{16}(OCH_3)_2N_2O$) and diethyl ($C_{16}H_{16}(OC_2H_5)_2N_2O$) ethers of haematoporphyrin are the only types of this group which have as yet been prepared. It is important to bear in mind the existence of these ethers and the fact that they are readily formed when haematoporphyrin in alcoholic solution is heated with mineral acids in order to explain the varying solubilities of the reduction products of haematin obtained by different observers. It has already been seen that the action of metals on alcoholic solutions of haemin and haematin containing hydrochloric acid has been largely used for the preparation of reduction products. It is necessary to remember the possibility of the formation of the diethylether of haematoporphyrin under these conditions.

As will be seen later, haematoporphyrin probably owes its feebly basic character to the presence of a pyrrol group.

Following upon these introductory statements, the chemical and spectroscopic characters of haematoporphyrin may be considered in some detail with the aid of the subjoined spectroscopic chart, which is mainly founded upon Garrod's observations. Although the

Taf. I.



α λ597–587
β λ576–565
γ λ557–541

Fig 1.

α λ623–613
β λ597–560
γ λ541–526
δ λ512–491

Fig 2

α λ625–616
β λ576–563
γ λ540–522
δ λ512–486

Fig 3

α λ586–570
β λ552–533

Fig 4

λ504–498

Fig 5

λ501–472

Fig 6

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the experimental data at present available are insufficient for the presentation of a review of the facts, which will apply to all cases and not be liable to revision; yet the following account may be regarded as representing the present state of our knowledge with regard to the relationships existing between the chemical and the spectroscopic characters of haematoporphyrin and its compounds. I have tried to combine with this description of the characters of haematoporphyrin and its compounds a comparison with those of the reduction products of haematin obtained by previous observers in order to ascertain the points in which the two kinds of pigment resemble or differ from one another.

(1) Spectrum of the salts of haematoporphyrin with mineral acids in presence of free mineral acid. This spectrum is best seen in alcoholic solutions of haematoporphyrin containing free hydrochloric or sulphuric acid. Two well marked absorption bands are seen on spectroscopic examination. The wave lengths of these bands are given in the chart. The band lying between D and E may be subdivided into three parts B, γ and a shaded area between these two parts of the band. γ is the most intense band then follow α and B. In dilute solution B is not visible as a distinct band and the spectrum is found

found to consist only of α and γ. The positions of the bands vary slightly according to the molecular weight of the fluid in which the haematoporphyrin is dissolved, the bands being displaced towards the red end of the spectrum the higher the molecular weight of the solvent. Farther addition of free mineral acid also moves the bands slightly towards the red end of the spectrum.

(2) Spectrum of the free (?) haematoporphyrin and its salts with metals of the alkalies. This spectrum shows four absorption bands α, B, γ and δ whose positions are given in the spectroscopic chart. Of these bands δ is the most intense, then follow γ, α and B. The two latter bands show almost an equal intensity of absorption. On acidification of an alkaline solution of haematoporphyrin showing these four bands the spectrum of acid haematoporphyrin appears. The addition of acetic or other organic acids leaves the alkaline spectrum unchanged. For the appearance of the spectrum of acid haematoporphyrin the addition of mineral acids is necessary.

(3) The so called alkaline spectrum showing five bands differs from that above described in showing in addition a narrow band in the red bordering upon C. Nencki and Sieber, Hammarsten, and Zoja regard this spectrum as that of the salts of haemato-

haematoporphyrin with mineral acids in the absence of excess of acid; while MacMunn considers the extra band in the red to be due to another pigment. Addition of mineral acid to its solution immediately results in the appearance of the spectrum of acid haematoporphyrin the "extra band" in the red vanishing along with α, B, γ and δ of the alkaline apectrum. This fact seems to be conclusive in favour of the view of Nencki and Sieber.

(4) The so called neutral spectrum of haematoporphyrin is probably to be regarded as a combination of the alkaline and acid spectra. It is seen when a salt of haematoporphyrin with a mineral acid is present along with an insufficient amount of free mineral acid to produce the acid spectrum. A solution of haematoporphyrin in amyl alcohol showing the four banded alkaline spectrum is converted into a solution showing the neutral spectrum on the addition of a trace of hydrochloric acid. On the other hand the addition of a trace of alkali to a solution showing the neutral spectrum causes the appearance of the four banded alkaline apectrum.

(5) The metallic spectrum of haematoporphyrin closely resembles that of oxyhaemoglobin. The B band is however darker and narrower than the corresponding band of haemoglobin. This spectrum is best

best seen in the case of the compound of zinc with haematoporphyrin. On addition of zinc chloride to an ammoniacal solution of haematoporphyrin the four banded alkaline spectrum becomes gradually converted into the two banded spectrum of metallic haematoporphyrin. Zoja has examined spectroscopically similar metallic compounds with calcium, barium, lead, mercury and tin. All these metallic compounds show a similar two-banded spectrum. The position and intensity of the two bands vary however with the metal used. Addition of mineral acid in slight excess to solutions of these metallic compounds at once causes the appearance of the spectrum of acid haematoporphyrin. There are also certain stable forms of metallic haematoporphyrin which are unaltered by acidification of their alcoholic solutions with hydrochloric acid. Saillet prepared an ammonium compound of haematoporphyrin by prolonged boiling of an ammoniacal solution of the pigment. The derivative so obtained closely resembles spectroscopically the metallic compound of zinc with haematoporphyrin; but possesses the same spectroscopic characters in acid as in alkaline or neutral solution. After being boiled with hydrochloric acid its solution shows the spectrum of acid haematoporphyrin.

haematoporphyrin.

A pigment has also been found by Hammarsten in the urine which, although apparently closely allied to haematoporphyrin, cannot be placed in any of the above mentioned groups.(25, Pt I) In acid and in alkaline solution it shows three bands, two between D and E similar in position and character to those of metallic haematoporphyrin, and one band between b and F. In acid solution the band between b and F is shifted slightly nearer F than in alkaline solution. On addition of ammonia and zinc chloride a well marked green fluorescence was developed. The latter fact would seem to indicate that the third band is due to admixture with urobilin and that the substance showing three bands on spectroscopic examination is really a mixture of urobilin with a substance showing two bands similar in position and character to those of metallic haematoporphyrin. *After remaining exposed to the air for two days in ammoniacal alcohol, the solution of the pigment was found to show the spectrum of alkaline haematoporphyrin. Hammersten believed that the conversion of this pigment into haematoporphyrin was due to the oxygen of the air. He regarded the pigment itself as a pre-

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* Hammarsten was, however, unable to convince himself of this by a separation of two substances from the solution showing three absorption bands.

precursor or mother substance of haematoporphyrin. The observations of MacMunn, Le Nobel, Nencki, and Sieber and Eichholz may now be reviewed in the light of the above mentioned facts.

(1) MacMunn's observations.- The spectroscopic characters of the reduction products of haematin in alkaline solution may first be compared with those of haematoporphyrin. In fig. 1 of spectral chart II the α band corresponds in position to the extra band in the alkaline spectrum of haematoporphyrin showing five bands. B corresponds closely in position to the α band of alkaline haematoporphyrin while δ and ε correspond closely to the γ and δ bands of alkaline haematoporphyrine. The γ band of the pigment described by MacMunn presents some differences from the B band of alkaline haematoporphyrin. These differences are probably due partly to the solutions examined being of different concentration, partly also to the presence of the band of unchanged alkali haematin which has become fused with the B band of alkaline haematoporphyrin. Figure 2 of spectral II chart shows the same pigment in acid solution. The α band of this "first reduction product" probably corresponds to the band of acid haematin in the red; while B and γ probably correspond to the α and γ bands of acid haematoporphyrin, the solution being

being too dilute to show a band corresponding to the B band of haematoporphyrin in acid solution. It is somewhat surprising to find a band between b and F at so early a stage of the reduction. It is improbable that any pigment resembling urobilin has as yet been formed. It is possible that the amount of acid added was insufficient to cause the disappearance of the $\mathcal{J}$ band, which corresponds to the most intense band of alkaline haematoporphyrin. Fig. 3 of spectral chart II showing the same pigment dissolved in rectified spirit will on comparison with Fig. 3 of spectral chart VII be found to closely correspond with the spectrum of neutral haematoporphyrin. As a result of this comparison the conclusion that MacMunn's "first reduction product" of haematin in alkaline solution consisted mainly of haematoporphyrin together with a small proportion of unchanged haematin, seems justified.

A comparison of Figs. 4 and 6 of spectral charts II & III showing the bands of the "second reduction product" in alkaline solution with the bands of alkaline haematoporphyrin shows that the two pigments are nearly identical in spectroscopic characters. In acid solution Fig. 5 of spectral chart II the pigment shows well the typical spectrum of acid haematoporphyrin without any band in the region of F, indicat-

indicating the absence of urobilin.

Spectrum 7 of chart III shows the spectrum of Mac-Munn's third reduction product in alkaline solution. It will be noted that the bands correspond in position to those of alkaline haematoporphyrin; but with the exception of the γ band they have faded considerably. Spectrum 8 of chart III shows the same pigment in acid solution. It will be noted that α and B correspond to the α and γ bands of a dilute solution of acid haematoporphyrin; while a well marked band is present in the region of F corresponding to the urobilin band. The conclusion that a pigment resembling urobilin is present is rendered more certain by the fact that an ammoniacal solution of the pigment showed a green fluorescence on the addition of zinc chloride; while the α band became simultaneously intensified. A solution of pure alkaline haematoporphyrin treated in the same way would in a short time have shown only two bands between D and E; while the α and β bands would have disappeared. Since the intensity of the band resembling that of urobilin increases pari passu with the decrease of the intensity of the bands of haematoporphyrin, it may probably be inferred that the urobilin is directly derived by the reduction of haematoporphyrin and the following rough scheme

scheme approximately represents MacMunn's results.

Haematin

|

Haematoporphyrin

|

Pigment resembling Urobilin.

The conversion of haematoporphyrin into urobilin was never even approximately complete, the greater part of the haematoporphyrin remaining apparently unchanged.

Review of the Results obtained by MacMunn by the Reduction of Haematin in Acid solution.

Spectrum 1 of chart IV is evidently that of acid haematoporphyrin plus some other pigment showing a band γ 529-469, while spectrum 2 is the five banded spectrum of alkaline haematoporphyrin. The first reduction product is therefore haematoporphyrin and possibly a pigment spectroscopically resembling urobilin.

In Fig. 3 of spectral chart IV the reduction is seen to have reached a later stage. The α and γ bands (α and B in the plate showing the pigment prepared by MacMunn) of acid haematoporphyrin are fainter and narrower, the band in the region of F has become darker and better defined. These facts would indicate that the first formed haematoporphyrin is diminishing in amount while a distinct increase in the pigment resembling urobilin is observable. Figure 4 of spectroscopic chart (4) shows the same pigment dissolved in ammoniacal alcohol. The spectrum is evidently that of the five banded alkaline haematoporphyrin. It is noteworthy that in the alkaline solution the band of the urobilinoid pigment fuses with the E band of alkaline haematoporphyrin so that it is no longer possible to tell that two pigments are probably present. The view that a

a urobilinoid pigment is present in this "the second reduction product" is also favoured by the fact that the addition of zinc chloride to the ammoniacal solution produced a faint green fluorescence.

The solution containing "the third reduction product" at first was yellow, and at first showed faintly a band at D apparently corresponding to the α band of acid haematoporphyrin, and a dark band at the blue end of the green apparently that of a urobilinoid pigment. On standing exposed to the air the solution became orange in colour and gave spectrum 5, which shows the α and γ bands of acid haematoporphyrin (α and B in the figure) as well as a band at F (γ) closely corresponding in position to that of urobilin. Spectrum 6 closely resembles the spectrum of the five banded alkaline haematoporphyrin; but is not perfectly typical, some of the bands approximating in position and character to those of neutral haematoporphyrin; while spectrum 7 ch. 5 (so-called alkaline urohaematoporphyrin) shows typically the features of the five banded alkaline haematoporphyrin. It is again noteworthy that the spectroscopic examination of an alkaline solution does not reveal the presence of the urobilinoid pigment owing to the 5th band of the alkaline haematoporphyrin spectrum being almost identical in position with that

that of urobilin so that the two bands have become fused. On adding zinc chloride to this solution a green fluorescence developed and solution showed spectrum 8. The spectroscopic characters of this solution are somewhat difficult to explain. Probably it shows a transition stage in the transformation of the five banded alkaline haematoporphyrin spectrum into the two banded metallic spectrum. In Fig. 9 this transformation is seen to be more nearly complete. The B and γ bands in the figure are in the position of the α and B bands of metallic haematoporphyrin while the α band of the haematoporphyrin has become fainter. Hammarsten (25 Pt. I) has shown that a solution of alkaline haematoporphyrin usually requires to stand several hours after the addition of zinc chloride before the metallic spectrum has fully developed. If the solution of haematoporphyrin be pure no green fluorescence is developed and the band of alkaline haematoporphyrin in the green blue completely disappears, instead of becoming accentuated as in the case of the pigment or rather mixture of pigments described by MacMunn. Apparently MacMunn had not allowed a sufficient interval to elapse for the full development of the metallic spectrum. The results obtained by MacMunn may be represented in the following scheme

scheme

Haematin

Haematoporphyrin

Chromogen of haematoporphyrin
in part converted into

a pigment resembling Urobilin.

The urohaematoporphyrin described by MacMunn is probably a mixture of haematoporphyrin with a pigment closely resembling or identical with urobilin.

In considering Le Nobel's results little need be added to the above discussion of MacMunn's work. By allowing the earlier stages of the reduction to take place at room temperature, Le Nobel was able to obtain haematoporphyrin practically free from any pigment resembling urobilin. The haematoporphyroidin described by Le Nobel appears to have been practically identical with haematoporphyrin. The so-called "isohaematoporphyrin" was simply haematoporphyrin in alkaline solution showing the five banded spectrum, and on treatment with sufficient excess of mineral acid it was converted into acid haematoporphyrin. The so-called urobilinoidin appears to have been a mixture of a chromogen of haematoporphyrin with a pigment closely resembling urobilin.

Nencki and Sieber(8) carried the reduction to a farther stage than either Le Nobel or MacMunn, and

and appear to have obtained a pigment closely resembling urobilin, and containing mixed with it less of the chromogen of haematoporphyrin. The hexahydrohaematoporphyrin described by Nencki and Sieber appears to have consisted of a stable form of metallic haematoporphyrin plus a pigment resembling urobilin. Its insolubility in alkalies indicates that it was related to the diethyl ether of haematoporphyrin. The haematoporphyrin molecule had probably undergone etherification owing to prolonged heating in an alcoholic solution containing hydrochloric acid. Eichholz *loc. cit.* succeeded in carrying the reduction farther than any of the previous observers. He obtained a pigment closely resembling urobilin and apparently free from admixture with chromogens of haematoporphyrin. By reduction of the pigment resembling urobilin he was also able to obtain a colourless chromogen of urobilin showing no absorption bands on spectroscopic examination. This chromogen on exposure to the air was again transformed into the pigment resembling urobilin. The following scheme may therefore be given as representing the complete reduction of haematin in acid solution.

Haematin
|
Haematoporphyrin in its various forms
|
Chromogen of haematoporphyrin
|
Urobilin
|
Chromogen of urobilin.

urobilin.

In alkaline solution it appears to be impossible to carry the reduction to its final stage. The final products of reduction in alkaline solution appear to be a mixture of haematoporphyrin and its chromogen with urobilin. To the above scheme must be added the fact that the solution containing the pigment resembling urobilin on long standing showed a band in the orange and yellow λ 598-570.

3.- Action of strong mineral acids on Haematin or Haemin.

(1) Action of strong sulphuric acid.

Although it is improbable that concentrated sulphuric acid acts partly as a reducing agent in the conversion of haematin into haematoporphyrin, it is necessary to consider its action on haematin partly because it was the first agent used in the preparation of haematoporphyrin from haematin and partly because its exact mode of action is still not quite clear.

Mulder and van Goudoever were the first to study * the action of concentrated sulphuric acid upon haematin. They dissolved haematin in the concentrated acid, allowed the solution to stand for some time exposed to the air and then poured it into excess of water. Bubbles of hydrogen gas (?) were evolved as the solution mixed with the water, and a precipitate of a pigment, which was later found to be iron free, formed. They believed that the decomposition was to be explained in the following manner. The sulphuric acid first united with the haematin. The compound so formed was decomposed on being poured into water, the iron being detached as ferrous sulphate and the hydrogen, displaced from the sulphuric acid by the iron, set free.

* Journal f. prakt. Chemie 1844 Bd 32 S 186

free.

Hoppe Seyler(1) farther studied this action and obtained results conflicting in some respects with the previous observations. He found that if haematin be mixed with concentrated sulphuric acid in a vessel over mercury, no development of gas took place, so that by the first action of sulphuric acid on haematin no hydrogen is evolved. If therefore ferrous sulphate be formed in the first instance the amount of hydrogen equivalent to the iron of the haematin must have united with the iron free residue. If the solution be next filtered through asbestos and then poured into excess of water, gas is evolved. This gas was found to consist not of hydrogen, but of a mixture of nitrogen and oxygen, the evolution of these being probably due to the evolution of the air dissolved in the water owing to the heat developed by its admixture with the concentrated sulphuric acid. Hoppe Seyler calculated that the amount of hydrogen equivalent to the iron in one gramme of haematin would occupy 38.6 cc at 0° C and 760 mm pressure. In one experiment he collected the gas developed on pouring the product of the action of concentrated sulphuric acid upon 1 gm. of haematin into water, and found that it amounted only to 5 ccm., and did not contain a trace of hydrogen. The

The precipitate of pigment obtained by pouring its solution in concentrated sulphuric acid into water was purified by solution in alkali and reprecipitation with dilute acid. Hoppe Seyler gave to it the name haematoporphyrin and described its spectroscopic characters in acid and in alkaline solution. He found that it contained sulphur in addition to carbon, hydrogen, nitrogen and oxygen. It was found impossible to obtain the pigment free from sulphur by repeated solution in dilute caustic potash and precipitation with dilute hydrochloric acid. Hoppe Seyler proposed as the probable formula, after subtraction of SO_3 $C_{68}H_{74}N_8O_{12}$, and gave the following equations to explain the action.

$C_{68}H_{70}N_8Fe_2O_{10} + 4H_2So_4 + O_2 = C_{68}H_{70}N_8O_{10}(H_2SO_4)_2 +$
$2\,Fe\,SO_4\ 2H_2O$ (Haematin)

On addition of water in excess

$C_{68}H_{70}N_8O_{10}(H_2SO_4)_2 + 2H_2O = C_{68}H_{74}N_8O_{12} + 2H_2SO_4$

It will be noted that the first equation assumes the presence of free oxygen to be necessary for the reaction. In accordance with this assumption Hoppe Seyler found that haematoporphyrin was only formed in considerable quantity by the action of strong sulphuric acid on haematin when the solution was freely exposed to the air. In absence of oxygen there is formed another iron free pigment--haematotin--which

which appears to be an addition compound of hydrogen with haematoporphyrin containing also less oxygen than haematoporphyrin. Hoppe Seyler gives as its probable formula $C_{68}H_{78}N_8O_7$.

Nencki and Sieber later studied the same reaction As a result of their analyses they gave $C_{32}H_{32}N_4O_5$ as the formula of haematoporphyrin and symbolised the action of strong sulphuric acid on haematin by means of the following equation

$$C_{32}H_{32}N_4FeO_4 + H_2SO_4 + O_2 = C_{32}H_{32}N_4O_5 + FeSo_4 + H_2O$$

(Haematin) (Haematoporphyrin)

It is difficult to understand how, under the influence of so powerful a dehydrating agent as strong sulphuric acid, a reaction of this kind involving the taking up of water could take place. Nencki and Sieber offer the following explanation. "The surprising phenomenon, that by the action of concentrated sulphuric acid haemin should take up water and be transformed into haematoporphyrin finds accord- to our hypothesis, its explanation in the fact that in the first instance esters with sulphuric acid are formed, which are decomposed by the action of water or alkalies." The existence of these esters which are supposed to precede the formation of haematoporphyrin, has not as yet been proved.

(2) Action of concentrated hydrochloric acid on

on haemin. If haemin be boiled with concentrated hydrochloric acid it apparently undergoes no chemical change; but if it be heated with concentrated hydrochloric acid in a sealed tube to 160° C., haematoporphyrin is formed. Dilute mineral acids are without action on haemin, unless as has already been seen, reducing agents be also present.

Nencki and Sieber(8) were the first to study comparatively the action of hydrochloric, hydrobromic,(9) and hydriodic acids on haemin dissolved in glacial acetic acid. They found that of these acids hydriodic produced the most complete decomposition of the haemin molecule, hydrobromic acid was next in activity; while hydrochloric acid had no action on haemin even at the boiling point of the glacial acetic acid. It is noteworthy that the degree to which these acids act upon haemin varies not with their strength as acids, but with their activity as reducing agents.

(3) Action of hydrobromic acid on haemin dissolved in glacial acetic acid.

In 1888 Nencki and Sieber(8) utilised the action of hydrobromic acid upon haemin dissolved in glacial acetic acid as a means for the preparation of haematoporphyrin. The process employed was the following. 5 gms. of haemin were gradually dissolved in

in glacial acetic acid which had been previously saturated with hydrobromic acid. The mixture was then heated until all the haemin had dissolved and gas had ceased to be evolved (about half an hour). The solution which had acquired a brilliant red colour was filtered and poured into large excess of water. A reddish brown precipitate formed, the fluid in which it was suspended still having a red tint. The precipitate was then filtered off. The haematoporphyrin in the filtrate may be almost completely precipitated by the addition of sufficient caustic soda to neutralise all the hydrobromic acid. At this stage the pigment being insoluble in dilute acetic acid separates out almost completely from the filtrate. The first formed precipitate and that obtained from the filtrate were then washed free from chlorides and bromides. From this crude haematoporphyrin Nencki and Sieber prepared the hydrochloride and the salts which have already been described. Free haematoporphyrin was prepared from the sodium salt by precipitation with acetic acid. The results of numerous analyses agreed well with the formula $C_{16}H_{18}N_2O_3$. Nencki and Rotschy[10] later estimated the molecular weight of this haematoporphyrin by Raoult's method using phenol as a solvent and found that the above formula for it is the correct one. They also

also estimated the molecular weight of bilirubin and found that its formula was also $C_{16}H_{18}N_2O_3$. Viewing their previous work in the light of these results Nencki and Sieber concluded that the haematoporphyrin prepared by the action of concentrated sulphuric acid upon haematin or haemin was to be regarded as the anhydride of the above. The relation of the two may be expressed by the following equation

$$(C_{32}H_{18}N_2O_3)_2 = C_{32}H_{34}N_2O_5 + H_2O.$$

It will be noted that the latter formula for the haematoporphyrin prepared by action of strong sulphuric acid on haemin represents it as containing two more hydrogen atoms than in the formula originally proposed by Nencki and Sieber. The preparation of haematoporphyrin from haemin by the action of hydrobromic acid may be represented by the following equation

$$C_{32}H_{31}ClN_4FeO_3 + 2HBr + 3H_2O = 2C_{16}H_{18}N_2O_3 + FeBr_2 + HCl + H_2.$$

(Haemin)

Nencki and Sieber like Hoppe-Seyler were unable to detect hydrogen in the gases evolved either during the decomposition of haemin by sulphuric or by hydrobromic acid. They gave the following explanation. "Without having given direct proof, we hold the view that the hydrogen formed in the conversion of haemin into haematoporphyrin is not set free because it is

is absorbed by another part of the easily reducible haemin." Along with haematoporphyrin a brown pigment was obtained by the decomposition of haemin by means of hydrobromic acid. This pigment was insoluble in dilute acids; but readily soluble in chloroform. Nencki and Sieber suggest that this may have been the reduction product the existence of which they had suspected. It was found impossible to obtain the pigment free from haematoporphyrin.

More recently Küster[29] has succeeded in separating from the products of the decomposition of haemin by hydrobromic acid, a pigment having the formula $C_{32}H_{36}N_4O_5$.
He suggests that the formation of this pigment explains the fate of the hydrogen which theoretically must be found during the conversion of haemin into haematoporphyrin and gives the following equations to explain the reaction

$$C_{32}H_{32}N_4FeO_4 + 2H_2O + 2HBr = 2C_{16}H_{18}N_2O_3 + FeBr_2 + H_2$$
(Haematin) (Haematoporphyrin)

The hydrogen set free in this reaction reduces part of the haematoporphyrin thus

$$2C_{16}H_{18}N_2O_3 + H_2 = H_2O + C_{32}H_{36}N_4O_5.$$
(Haematoporphyrin)

Recently Nencki and Zaleski[11] have found that a better yield of haematoporphyrin may be obtained by

by the above method, if the solution of haemin in glacial acetic acid saturated with hydrobromic acid be allowed to stand exposed to the air at room temperature for three days instead of being heated to the boiling point. The yield is nearly quantitative. From 10 gms. of haemin they obtained 9.1 gms. of haematoporphyrin. The very fact that the yield of haematoporphyrin by this method is so good increases the difficulty of explaining the fate of the hydrogen which must be formed. It has already been seen that haematoporphyrin on reduction is transformed into a chromogen which on exposure to the air is transformed again into haematoporphyrin. Hoppe Seyler, as has already been stated, showed that in the reduction of haematoporphyrin by metals plus mineral acids the process essentially consisted in the formation of addition products of haematoporphyrin with hydrogen, no oxygen being withdrawn from the molecule. It may therefore be concluded with some probability that haematoporphyrin readily forms unstable addition products with hydrogen (chromogens) which on exposure to the air yield haematoporphyrin. Although no direct proof is yet available, I should therefore suggest the possibility that the formation of haematoporphyrin by the action of hydrobromic acid on haemin takes place according to the two following

following equations.

$$C_{32}H_{32}N_4FeO_4 + 2H_2O + 2HBr = 2C_{16}H_{19}N_2O_3 + FeBr_2$$

(Unstable addition product of hydrogen with haematoporphyrin)

$$2C_{16}H_{19}N_2O_3 + \underline{O} \text{ (of the air)} = 2C_{16}H_{18}N_2O_3 + H_2O.$$

(Haematoporphyrin)

In order to test the truth of this hypothesis it would be necessary to carry out the preparation of haematoporphyrin by Nencki's method in a hydrogen atmosphere. The same difficulties would therefore have to be overcome as in the preparation of haemochromogen by Zeynek's method.

(4) Action of hydriodic acid on haemin dissolved in glacial acetic acid.

By the action of hydriodic acid on haemin dissolved in glacial acetic acid Nencki and Sieber obtained a body resembling Urobilin in addition to amorphous pigments containing iodine. This change took place in the cold. It is obvious, therefore, that hydriodic acid produces a much more complete decomposition of the haemin molecule than hydrobromic acid.

4.- Action of hydriodic acid and phosphonium iodide on haemin dissolved in glacial acetic acid.

The aim of Nencki and Zaleski[a] in using this method was to try to obtain phylloporphyrin, a derivative of chlorophyll closely resembling haematoporphyrin in spectroscopic characters; but differing from it in containing only one atom of oxygen. They thus hoped to establish a chemical relationship between haematoporphyrin and phylloporphyrin. 5 gms. of crude haemin were digested on the water bath for 20 minutes with 40 ccm. of hydriodic acid (sp. gr. 1.74) and 75 ccm. of glacial acetic acid. 3 gms. of phosphonium iodide were then gradually added. After heating for a farther 15 minutes the colour of the fluid had become clear red with a yellowish tint when examined in a thin layer. When a small test portion of the fluid ceased to yield a precipitate on dilution with an equal volume of water the reaction was completed. A red precipitate formed on pouring the fluid diluted with an equal volume of water into a large excess of water (1 - 1.5 litres). The quantity of the precipitate was increased by the addition of sufficient caustic soda to neutralise all the free hydriodic acid. The precipitate was next filtered off and washed free from chlorides and

and iodides. It was then suspended in 1.5 litres of boiling water and brought into solution by the addition of hydrochloric acid until the aqueous solution containing 2.5 per cent of the acid. The pigment is insoluble in greater concentration of the acid. The dark violet solution thus obtained was filtered and then concentrated on the water bath until the surface of the solution became covered with minute brownish red microscopical needles. The crystals so obtained were mixed with a considerable amount of amorphous material. They were purified by washing with 5% hydrochloric acid, and repeated recrystallisation from hot 2.5% hydrochloric acid. A preparation, which had been recrystallised four times was found to be free from iron, iodine, and phosphorus and contained no amorphous material. An analysis of the crystals dried to constant weight gave a result agreeing with the formula $C_{16}H_{18}N_2O_2$ HCl. Nencki and Zaleski therefore believed that they had obtained the hydrochloride of a substance intermediate between haematoporphyrin and phylloporphyrin to which they gave the name mesoporphyrin so as to indicate the probable chemical relationship of the three bodies. This relation may be represented by the three formulae

$C_{16}H_{18}N_2O_3$ (Haematoporphyrin)

$C_{16}H_{18}N_2O_3$ (Haematoporphyrin)

$C_{16}H_{18}N_2O_2$ (Mesoporphyrin)

$C_{16}H_{18}N_2O$ (Phylloporphyrin)

From the hydrochloride free mesoporphyrin was obtained by solution in alkali and precipitation with dilute acetic acid. Mesoporphyrin is spectroscopically identical with haematoporphyrin and resembles it closely in its solubilities. Heated in a capillary tube it does not melt even at a temperature of over 340° C. The yield of mesoporphyrin is comparatively small amounting to about 20 per cent of the haemin used. A substance containing iodine and an amorphous reddish brown pigment are also formed.

Nencki and Zaleski next tried, by more prolonged heating of haemin dissolved in glacial acetic acid with a larger quantity of hydriodic acid and more phosphonium iodide, to withdraw another atom of oxygen from haematoporphyrin and so obtain phylloporphyrin. They failed to obtain phylloporphyrin; but succeeded in separating a body free from oxygen and volatile with watery vapour. The reduction fluid after being boiled for half an hour was diluted with four to five times its volume of water, and placed in a distillation flask connected with a condenser. Sufficient caustic soda to neutralise all the hydriodic acid and the greater part of the acetic acid

acid was then added from a stoppered funnel. The mixture was then heated to the boiling point. In the first portions of the distillate a colourless oily substance was present having an odour recalling that of both scatol and naphthaline. This is only slightly soluble in water. It colours red a pine wood strip dipped in hydrochloric acid, the latter fact indicating that it is a pyrrol derivative. Its dilute watery solution gives a white amorphous precipitate with mercuric chloride. This precipitate is soluble in alcohol but insoluble in water. It was freed from excess of mercuric chloride by washing with water, dried in vacuo over sulphuric acid, and analysed. The results of the analyses indicated that the formula of this salt was $(C_8H_{12}N)_2 H_g(H_gCl_2)_2$. The volatile substance named by Nencki and Zaleski haemopyrrol also yielded a precipitate with picric acid in aqueous solution. This precipitate was crystallised from benzene. Analyses of the picric acid compound indicated that its formula was $C_8H_{13}N.C_6H_2(NO_2)_3OH$. From these results it was concluded that haemopyrrol has the empirical formula $C_8H_{13}N$. It is soluble in dilute mineral acids but insoluble in acetic acid. Crystalline salts with the former were not obtained. On exposure to the air haemopyrrol is converted into a

a red substance which appears to closely resemble, if not to be identical with, Urobilin. Its solution in ammonia is yellow and on addition of zinc chloride becomes fluid, and shows a well marked green fluorescence. On spectroscopic examination it shows a band in the position of urobilin.

If injected into the subcutaneous tissue of a rabbit it is excreted as urobilin. The greater part of the urobilin is found in the urine 3 - 10 hours after injection.

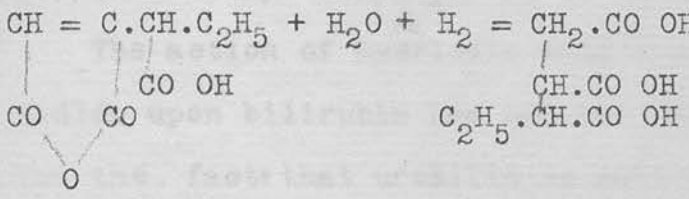
The constitution of haemopyrrol is still uncertain. One molecule of haemin is split almost quantitatively into two molecules of haematoporphyrin, and one might conjecture that on reduction haematoporphyrin yielded two molecules of haemopyrrol according to the equation

$$C_{16}H_{18}N_2O_3 \quad H_2 \quad 2C_8H_{13}N \quad 3H_2O.$$

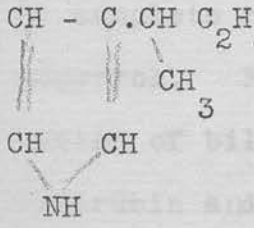
Since however 32 per cent of haemopyrrol is the maximum yield as yet obtained from haemin the truth of the above equation cannot be regarded as proved. When, however, the matter is viewed in the light of the results obtained by Küster by the oxidation of haemin and haematoporphyrin dissolved in glacial acetic acid by means of sodium bichromate it seems possible that one molecule of haematoporphyrin is capable of yielding on reduction two molecules of

of haemopyrrol. By the oxidation of both haemin and haematoporphyrin Küster obtained two acids $C_8H_9NO_4$ and $C_8H_8O_5$, which have probably the following constitution. The yield of these acids was about 50%.

From the latter acid Kölle has obtained by reduction with hydriodic acid, ethyltricarballylic acid.

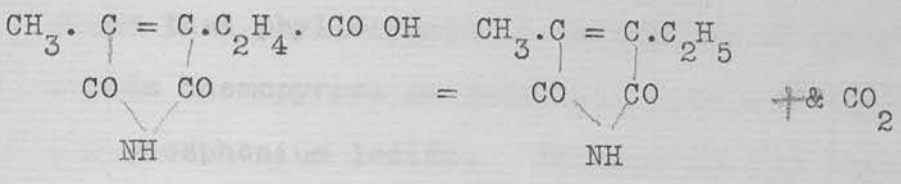


From these results it seems probable that haemopyrrol as a butyl pyrrol having the following structure

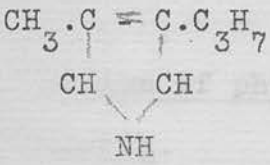


The above constitutional formula cannot be regarded as definitely proved. If carbon dioxide be split off from the imide $C_8H_9NO_4$, an imide $C_7H_9NO_2$ is obtained which Küster believes to be identical with the imide of methyl-ethyl-maleic acid.

acid.



In accordance with this view Nencki has suggested as an alternative formula for haemopyrrol



The evidence, therefore, points to haemopyrrol being either a methyl-ethyl-pyrrol or an isobutylpyrrol.

The action of hydriodic acid and phosphonium iodide upon bilirubin has not yet been investigated; but the facts that urobilin is derived from bilirubin and that haemopyrrol on oxidation yields a substance closely resembling urobilin render it probable that on complete reduction bilirubin would also yield haemopyrrol. Further, Küster has obtained by the oxidation of bilirubin the two acids above described. Bilirubin and haematoporphyrin are therefore not only isomeric, but also closely related to one another in chemical constitution. Nencki and Zaleski have suggested two possible formulae for haematoporphyrin but the evidence is still insufficient.

In a more recent paper Nencki and Marchlewski[1] have shown that phyllocyanin, a derivative of chlorophyll yields haemopyrrol on reduction with hydriodic acid and phosphonium iodide. Chlorophyll and haemoglobin may therefore be regarded as chemically related substances.

Action of phosphorus pentachloride upon haematoporphyrin.

Nencki and Zaleski *loc. cit.* also tried to obtain phylloporphyrin from haematoporphyrin by the action of phosphorus pentachloride. They hoped by this means to replace the two hydroxyl groups of haematoporphyrin by chlorine, and then to substitute hydrogen for the two chlorine atoms in accordance with the following equations.

$$C_{16}H_{18}N_2O_3 + 2\,PCl_5 = C_{16}H_{16}Cl_2N_2O \;\&\; 2\,POCl_3 \;\&\; 2\,HCl$$

$$C_{16}H_{16}Cl_2N_2O + 2\,H = C_{16}H_{18}N_2O + 2\,HCl$$

(Phylloporphyrin)

The reaction however went much farther than they had anticipated, the only product being a pigment resembling urobilin in its spectroscopic characters.

Electrolysis of an alkaline Haematin solution.

By the electrolysis of alkaline haematin solu-

solutions Zeynek finally obtained a colourless fluid in which was suspended ferric hydrate. He was unable to detect either haemochromogen or haematoporphyrin amongst the products of decomposition. The electromotive force of the currents used varied from 4-10 volts. Zeynek unfortunately gave no farther details with regard to the conditions of the experiment.

Decomposition of haemoglobin in an atmosphere free from oxygen.

Instead of reducing pure haematin prepared from oxyhaemoglobin one may study the pigments produced by the decomposition of reduced haemoglobin by means of acids and alkalies. Hoppe Seyler[1], who was the first to study these products, employed a somewhat complicated apparatus for their preparation. The following description gives an outline of the end aimed at and the results obtained. An account of the experimental details would be unnecessary. A solution of pure oxyhaemoglobin free from methaemoglobin was freed from oxygen, free and combined by passing a current of pure hydrogen through it for two to three hours. The solutions of the reagents used for the decomposition of the haemoglobin were

were also freed from oxygen in a similar manner, and finally the latter were mixed with the solution of haemoglobin in a hydrogen atmosphere.

On mixing a solution of haemoglobin with alcohol acidified with sulphuric acid in the absence of oxygen, a red precipitate forms. On warming the solution the precipitate becomes decolourised while the fluid acquires a purple red colour and shows four absorption bands two between C and D and one broad very dark band between D and E. Of the two bands between C and D that lying nearer to C is identical in position with the band of haematin in alcohol acidified with sulphuric acid and is probably due to the fact that the solution of haemoglobin had also contained either methaemoglobin or a little oxyhaemoglobin. A faint broad fourth absorption takes up the space lying between b and F. Hoppe Seyler at first thought that the substance showing in solution the above described absorption bands was haemochromogen in acid solution. Although the exact position of the bands is not given, Hoppe Seyler's description of their characters and approximate position leads one to believe that they were due to a mixture of acid haematin with acid haematoporphyrin. This view was afterwards advanced by Jaderholm (25) Hoppe Seyler seems later to have recognised that the

the above described four banded spectrum is not that of haemochromogen in acid solution. The following description taken from his text-book of physiological chemistry (pages 394-395) gives his most recent published account of this subject. "If a haemoglobin solution be decomposed by means of dilute acid into proteid and haemochromogen in the presence of oxygen, haematin is likewise formed, while, in the absence of oxygen, the first formed pigment (haemochromogen) rapidly undergoes decomposition into a less sensitive colouring matter, haematoporphyrin, ferrous salts being simultaneously formed. Solutions containing haemochromogen in acid solution absorb in least degree the red and orange of the spectrum up to D, thereafter the green blue, while between these regions from D to E lies a broad darker part. At no degree of dilution can sharply limited absorption bands be observed in an acid haemochromogen solution."

On decomposition of haemoglobin by means of dilute alkali in the absence of oxygen, Hoppe Seyler obtained haemochromogen in alkaline solution showing the two bands already described as those of Stokes "reduced haematin."

PART III.

Methods used for the preparation of Haematin.

Haematin appears to be best prepared from its chlorine compound haemin by decomposition of the latter by means of dilute alkali. A difficulty, that meets one at the outset, is to decide as to which of the various methods for the preparation of haemin yields the purest product. Almost as many formulae have been suggested for haemin and haematin as there are methods for their preparation. It appears from the work of Hoppe Seyler, Nencki and Sieber, Küster and Morner, that these differences in the composition of the product obtained depend upon the fact that haemins of different composition and structure are formed according to the conditions under which the preparation is carried out. The following are the chief formulae for haemin which have been proposed.

$C_{34} H_{35} N_4 Fe O_5 HCl$ (Hoppe Seyler)

$C_{32} H_{31} N_4 Fe O_3 Cl$ (Nencki and Sieber)

An even larger number of formulae has been proposed for haematin. The chief of these are the following: $C_{34} H_{35} N_4 Fe O_5$ (Hoppe Seyler) $C32 H_{32} N_4 Fe O_4$ (Nencki and Sieber), $C_{32} H_{34} N_4 Fe O_3$ (Küster and Hufner), $C_{35} H_{36} N_4 Fe O_3$ (K. Mörner). All these observers agree that for one atom of iron the mole-

molecules of haemin and haematin contain four of nitrogen. I have omitted to mention the older formulae and have only given those which the observers mentioned have proposed as a result of their more recent researches. Hoppe Seyler in his earlier papers suggested formulae different from that given above. In this paper I shall only describe in full the methods which I have myself employed.

For the preparation of haematin, I at first employed the following method suggested by Hoppe Seyler.* The proteids of defibrinated ox blood were precipitated by the addition of four to five volumes of 95 per cent alcohol. The fluid and precipitate were then left in contact with one another for at least twenty-four hours in order to ensure complete coagulation of the proteids. The precipitate was then filtered off under reduced pressure, washed with alcohol until the filtrate, which was at first light yellow, became colourless, and then finally washed twice with ether. I hoped by washing with alcohol and ether to remove the greater part of the fats, lecithin and cholesterin. The precipitate was then dried, finely powdered, and extracted with ether acidified with glacial acetic acid (four volumes of ether to one volume of glacial acetic acid). The dark brown solution of haematin thus obtained was

* Handbuch der physiol. chem. Analyse p 216

was then filtered. It is, as will be shown later, free from proteid, but probably contains some cholesterin and fats. The ether was then distilled off and a concentrated solution of haematin in glacial acetic acid thus obtained. This solution was used for my earlier experiments on the reduction products of haematin. From it haematin was prepared in the following way. The solution in glacial acetic acid was mixed with five times its volume of distilled water. Part of the haematin was thus precipitated. The precipitation was rendered more complete by partial neutralisation with dilute caustic soda. The precipitate so obtained was filtered off, washed with water, dissolved in two per cent sodium hydrate, and purified by reprecipitation with acetic acid.

I also used haematin prepared from haemin by *Hoppe Seyler's method. The chief difficulty met with in this process was to obtain the crystals free from proteid

* Hoppe Seyler's Zeitschrift für physiol. Chem. Analyse p. 218

Another method, which has been found to give a very good yield of haemin from blood, is that devised by Schalfijew. The chief objection to this method is that it requires the use of very large quantities of glacial acetic acid (four to five litres of glacial acetic to each litre of blood) which are somewhat difficult to recover from the mother liquid. Farther, unless the crude haemin so obtained be purified by recrystallisation from chloroform containing quinine, it is found to contain considerable quantities of proteid. The method is therefore a somewhat costly one for the preparation of large quantities of haemin. For these reasons I have not adopted it.

I also tried Mörner's method,[691] which has the advantage of being much less expensive. The proteids of one litre of defibrinated blood diluted with three volumes of water faintly acidified with sulphuric acid were either precipitated by heat or the undiluted blood was mixed with four to five times its bulk of 90-95 per cent of alcohol, and in the latter case allowed to stand for at least twenty four hours. The coagulum thus obtained was then well mixed with about two litres of alcohol acidified with either oxalic acid or 35 ccm. of a previously prepared mixture of 17.5 ccm. concentrated sulphuric

Hammarsten's Lehrbuch der physiologischen Chemie p 151 also note in Küster's paper no 29 of bibliography

sulphuric acid with 17.5 ccm. alcohol. The mixture was allowed to stand for an hour at room temperature. The fluid was then partially freed from the coagulum by filtration through well washed calico. The dark brown solution thus obtained was filtered, then heated to the boiling point. 8 ccm. of 25 per cent hydrochloric acid previously diluted with about 16 ccm. of 90 per cent alcohol were then added and the solution was allowed to cool. The solution after standing for two days was filtered off from the sediment of haemin crystals, which had separated out. The haemin crystals so obtained were then washed with water and dried. The crystals were however mixed with amorphous material. On trying to dissolve them in one per cent caustic soda solution I found that a large proportion of the haemin did not dissolve. I was unable to find a cause for the insolubility of the crystals in dilute alkali. I have since found that other observers including Mörner himself, as well as Nencki and Sieber, had occasionally obtained a similar result. Mörner, in a more recent communication, states that in order to secure haemin soluble in alkalies, it is necessary to heat the acid alcoholic solution rapidly to the boiling point and then to cool it rapidly by immersion of the vessel containing it in cold water. At the time at which I

I tried the method I was not aware of the need for these precautions. If the boiling be prolonged or the solution be allowed to cool slowly, the crystals which separate out are mixed with amorphous material and partially insoluble in dilute alkalies.

Another method which has been largely used for the preparation of haemin is that devised by Nencki and Sieber. It consists essentially in the extraction of oxyhaemoglobin precipitated by alcohol with amyl alcohol acidified with hydrochloric acid. I found, however, on consulting the literature of the subject that the yield of haemin obtained by this method is not good and that one is apt to obtain by its means a haemin insoluble in alkalies. For these reasons I have not tried the method.

The method of preparation which I have exclusively employed in my later work was recently suggested by Zeynek,(361) and employed by Nencki and Zaleski loc.cit. in a slightly modified form. By this method Nencki and Zaleski believe that one obtains a purer product than by any of the previous methods. The following description gives the details of the method as it was employed by Nencki and Zaleski. The sediment of red blood corpuscles obtained from ox blood by the method originally used by Hoppe Seyler is mixed with sufficient acetone to precipitate the proteids. After 24

24 hours the proteid coagulum is filtered off. Each 400 gms. of the coagulum thus obtained is then heated with 1300 ccm. of acetone. As soon as the acetone commences to boil, 35 ccm. of hydrochloric acid (sp. gr. 1.124) are added and the boiling continued for five minutes. The dark red brown solution of haemin is then filtered off. After the solution has stood for one to three days haemin crystals are found to have separated out on the bottom and sides of the vessel. These crystals were washed first with ether, then with 30 per cent acetone and finally with .1 per cent aqueous hydrochloric acid. Nencki and Zaleski were able to recrystallise the haemin from 80 per cent acetone containing .6 per cent of quinine. The crystals so obtained are well formed and completely free from amorphous material. Their crystallographic characters have been described fully by Zeynek. At first I followed precisely the directions given by Nencki and Zaleski, and obtained haemin in crystals absolutely free from admixture with haemin in amorphous form. I found, however, that the yield of haemin was small. I tried to get a better yield by first concentrating the haemin solution in acetone to half its original bulk before leaving it to crystallise. By this method I obtained a much better yield, and

and the crystals were found to be free from admixture with haemin in the amorphous form.

The crystals obtained by the first method were mainly long, fine, hair-like needles frequently slightly curved. The size of the needles varies according to the rapidity with which the haemin crystallises out. By the modified method I obtained larger crystals showing the rhombic form in addition to the needles above described. In addition to the haemin crystals there are also found the typical plates of cholesterin. I further identified the cholesterin by means of the usual tests. For the separation of the cholesterin I employed the following method. 100 ccm. of the haemin solution in acetone were evaporated to dryness, and then extracted with ether. The ethereal solution dissolved some of the haemin as well as cholesterin and acquired a reddish brown tint. The ethereal solution was filtered and shaken up with dilute aqueous solution of sodium carbonate in a separating funnel. The haemin passes in the form of alkaline haematin into the aqueous solution while the ethereal solution becomes decolourised. The ethereal solution was then separated from the aqueous layer, and on evaporation yielded a yellow residue. Part of the residue was dissolved in chloroform and mixed with an equal vol-

volume of concentrated sulphuric acid. The chloroform rapidly acquired a red colour and the subjacent sulphuric acid showed a well marked green fluorescence. Another portion of the residue was found to give Liebermann's reaction for cholesterin.

I next tried the following modification of Nencki and Zaleski's method in order to obtain a preparation of haemin free from cholesterin and fats, the aim of the following somewhat tedious process being to free the proteid coagulum as thoroughly as possible from adherent fats and cholesterin. The sediment of red blood corpuscles obtained by centrifugalising oxalate plasma of ox's blood was coagulated by alcohol, and allowed to remain in contact with the alcohol for at least 24 hours. Since about two-thirds of the proteids of blood consist of haemoglobin, I have frequently precipitated all the proteids from defibrinated blood by the addition of four to five times its volume of 90 per cent alcohol, instead of using the sediment of red blood corpuscles from oxalate plasma. The proteid coagulum was next filtered through well washed calico, dried, and finely powdered. The powder was then mixed with about five times its bulk of ether, and boiled for an hour on the water bath in a flask provided with a reflux condenser. The fluid was then allowed to

to cool and the coagulum was again filtered through calico. The ethereal solution was next filtered, placed in a distillation flask and the ether distilled off, the distillate being again used for the extraction of the proteid coagulum. The residue left in the distillation flask was found to contain both fat and cholesterin. The extraction with ether was twice repeated. The powder was then boiled with neutral acetone which was filtered off. Finally the powder was boiled with acetone containing hydrochloric acid, in the proportions used by Nencki and Zaleski for 20 minutes. The fluid was then filtered. The dark red solution of haemin in acid acetone was next placed in a thick-walled glass vessel and concentrated by distillation to about a fourth of its original bulk. The distillation was carried out under slightly reduced pressure, the water bath being kept at a temperature between 35° and 40° C. with the object of lessening the risk of decomposition of the haemin. The concentrated solution of haemin in acetone was then allowed to stand for several days in a closed vessel. Haemin was found to have separated out in considerable quantities on the sides and bottom of the vessel. On microscopical examination the crystals were found to be well formed, free from amorphous material and cholesterin

cholesterin and readily soluble in dilute alkalies. The crystals were next filtered off, washed with water and ether and then dried. By this method I have prepared about 20 gms. of haemin. The yield is not very good as the mother liquor, from which the crystals separate out, contains considerable quantities of haemin. On trying to obtain haemin from the filtrate by farther concentration, I found that the haemin crystals so obtained were mixed with amorphous material. From a litre of blood I was only able to obtain as an average amount about 1.5 gm. of pure haemin crystals; but the method has the great advantage that the solvents used may be recovered fairly completely and re-utilised for further experiments.

In conclusion, the nature of the different forms of haemin prepared by the above described methods may be shortly discussed. Nencki and Zaleski have found that the haemin molecule contains two hydroxyl groups, the hydrogen atoms of which may be readily replaced by alkyl and acid radicals, and have been able to prepare several of these ethereal compounds. The following is a very brief account of their results, which are of much importance for the explanation of the different forms of haemin obtained by different observers. They believe that free haemin

haemin has the following formula:-
$C_{32}\ H_{29}\ (OH)_2\ N_4\ O\ Fe\ Cl$. Haemin, although frequently named the hydrochloride of haematin, is not a salt obtained by the addition of hydrochloric acid to the haematin molecule. If haemin be treated with dilute alkali the chlorine atom is replaced by a hydroxyl group-

$C_{32}\ H_{29}\ (OH)_2\ N_4 O\ Fe\ Cl + NaOH = C_{32}\ H_{29}\ (OH)_2\ N_4 O\ Fe\ OH + NaCl$.

If haemin be dissolved in methyl alcohol saturated with hydrochloric acid, the dimethyl ether of haemin is formed having the following formula -

$C_{32}\ H_{29}\ (OCH_3)_2\ N_4 OFe\ Cl$.

The diethyl ether of haemin may be prepared in a similar manner from its solution in ethyl alcohol. Both those ethers are insoluble in aqueous alkalies. On boiling with aqueous alkalies they are slowly saponified and dissolved. The monomethyl and monethyl ethers ($C_{32}\ H_{29}\ (OH)\ (OCH_3)$) are soluble in dilute alkalies. Nencki and Zaleski believe that the haemin prepared by the amyl alcohol method is the monamyl ether of haemin having the following formula:-

$$C_{32}\ H_{29}\ (OH)\ (OC_5H_{11})N_4\ O\ Fe\ Cl.$$

If the solution be heated for a longer period the diamyl ether of haemin is formed which is insoluble

insoluble in alkalies. The haemin prepared by Morner's method is the monethylether. In this case also if the solution in acid alcohol be heated too long the diethylether is formed which is insoluble in dilute alkalies. This affords an explanation of the results previously described. Nencki and Zaleski regard the haemin prepared by Schafejeff's method as a compound of free haemin with acetyl. From it mono- and di-methyl ethers may be prepared so that the acetyl group does not take the place of either of the two hydroxyl groups, upon which the solubility of haemin in alkalies depends. The haemin prepared by crystallisation from acetone is probably free haemin having a percentage composition agreeing with the formula $C_{32} H_{31} N_4 O_3 Fe Cl$.

Results obtained by the reduction of haematin dissolved in glacial acetic acid.

All previous observers had used mineral acids plus metals as reducing agents. I thought that by making use of organic acids, one might make the reduction less rapid and be able to trace more thoroughly the stages of the reduction process and at the same time ascertain whether any substances were formed intermediate between those already described. One of the chief obstacles to the study of the products of the reduction of haematin lies, as has already been seen, in the fact that they readily undergo oxidation on exposure to the air. It seemed therefore desirable to obtain some reducing agent which might be not only gradual in its action, but also allow one to obtain the products of its action in a form resistant to the oxidising action of the air. Unstable products of reduction have in some cases, notably that of indigo white, been obtained as stable acetyl derivatives by combining acetylisation with reduction. Since, as has already been seen, haemin and haematoporphyrin readily yield derivatives with alcoholic and acid radicles, I thought that the combination of acetylisation with reduction might possibly yield interesting results in the case of the reduction products of

of haematin. Simce haemin and haematoporphyrin both readily yield acetyl derivatives I thought that glacial acetic acid might prove a sufficiently powerful agent for the acetylisation. As will be seen later, I have also tried a mixture of acetic anhydride with sodium acetate for the same purpose.

My earlier experiments were mainly tentative being devised for the purpose of finding out whether the subject would repay farther work; but as the form of haematin used was prepared by a different method than that adopted later, and the results obtained differ in some minor points from those of my later experiments, I shall give a brief sketch of my earlier work. A small direct vision spectroscope without any provision for accurately localising the position of the absorption bands was used, and their position could therefore only be given approximately by a reference to Fraunhofer's lines.

A solution of haematin in glacial acetic acid prepared by the method first described was employed for all the experiments. The metals used were pure zinc and aluminium both in the form of powder. I shall first describe the action of the aluminium.

At the temperature of the room aluminium appeared to have no action on haematin dissolved in glacial acetic acid. A solution which had been in a stop-

stoppered bottle for 24 hours still showed the red brown colour and spectroscopic characters of acid haematin. The same solution was then heated in a loosely corked vessel to 60° C. for two hours.

The solution was then bright red in colour. After being filtered, it showed on spectroscopic examination a faint band in the red, about midway between C and D and two well marked bands between D and E, closely resembling the bands of metallic haematoporphyrin in position and character. The band nearer D is narrower and darker than the one near E. On adding hydrochloric acid to the solution no alteration was noted in its spectroscopic characters.

If the solution of haematin in glacial acetic acid be boiled with a small quantity of aluminium for about five minutes it acquires a bright red colour appearing pink when examined in a thin layer. On boiling for fifteen minutes longer no alteration in colour took place. The solution was then filtered and showed, on spectroscopic examination, the two bands already described. When examined in a thicker layer it shows also a faint band in the red about midway between C and D. The solution was then poured into a separating funnel diluted with about three times its volume of water and then re-

repeatedly shaken with amyl alcohol. The latter solvent takes up all the pigment, leaving the subjacent aqueous layer colourless. The aqueous layer was separated from the alcohol and acidified with hydrochloric acid. On addition of potassium ferrocyanide the solution became blue, and a precipitate of prussian blue gradually separated out. During the process of reduction iron is therefore split off from the haematin molecule. I next prepared a solution of the pigment in acetone by a method founded upon the fact that acetone, although miscible with water in all proportions, is insoluble in saturated solutions of ammonium sulphate. The solution of the reduction product in glacial acetic acid was diluted with three times its volume of water. The dilute solution was saturated with ammonium sulphate and then extracted with acetone. The acetone rose to the surface and was found to have dissolved out all the pigment, leaving the subjacent watery solution colourless. The two layers were separated by means of a separating funnel. On spectroscopic examination the solution in acetone was found to show the two absorption bands already described. The acetone solution of the pigment was then diluted with an equal volume of water and placed in a porcelain basin on the water bath. As the acetone gradually

gradually evaporated the pigment separated out in the form of a red precipitate which, on microscopical examination, was found to be amorphous. Sufficient concentrated hydrochloric acid was added to make a solution of about 5%. The greater part of the pigment remained undissolved so that it was only possible to obtain a dilute solution in 5 per cent aqueous hydrochloric acid. On spectroscopic examination the pigment was found to show two absorption bands between D and E apparently identical in position and character with those already described. Even after boiling for twenty minutes the acid solution retained its spectroscopic characters apparently unaltered. The solution of the pigment in acetone was transferred to an alkaline solution by a method similar to that above described. The pigment is readily soluble in one per cent alkali and on spectroscopic examination shows the two bands between D and E. In a thicker layer it also shows a faint band in the red between C and D. The most characteristic feature of the pigment above described is the remarkable uniformity of its spectroscopic characters in neutral, in acid, and in alkaline solutions. The pigment is also readily soluble in chloroform and ethyl alcohol.

Effect of more prolonged heating of haematin dissolved in glacial acetic acid with Aluminium.

Haematin dissolved in glacial acetic acid was boiled with aluminium powder for four hours in a flask provided with a reflux condenser. The solution was then filtered. It had a light yellow colour and no absorption bands were noted on spectroscopic examination. After standing exposed to the air for a few minutes the upper layers of the solution acquired an orange tint. After standing for an hour the solution had become reddish brown with a slight green tint when examined in a thin layer. On spectroscopic examination four absorption bands were noted, two well marked narrow bands lying in the red between C and D, and two bands between D and E apparently identical in position and character with the bands of the first reduction product already described. The solution was then diluted with three volumes of water and extracted with chloroform. The chloroform solution took up a red brown pigment while the supernatant aqueous layer had a light olive green tint. On spectroscopic examination the chloroform solution showed the two bands between D and E, and also the two narrow bands in the red between C and D. The latter, however, were much more indistinct than in the original solution. The aqueous layer

layer containing the olive green pigment was too dilute to show any absorption bands on spectroscopic examination. The aqueous layer after it had been separated from the chloroform by means of a separating funnel was extracted with amyl alcohol. The latter solvent took up the greater part of the olive green pigment and on spectroscopic examination was found to show two well marked bands between C and D, as well as two faint bands between D and E. The latter bands were apparently identical in position with those of the first reduction product. The experiments above described seem to indicate that the original solution had contained the chromogens of two pigments. One was probably the chromogen of the first reduction product, while the other was that of a greenish pigment showing two bands between C and D. I did not succeed, however, in proving this by a separation of the pigments. As will be seen later on, I have succeeded in obtaining the green pigment by reduction of the first reduction product dissolved in glacial acetic acid by means of zinc dust.

Reduction of haematin dissolved in glacial acetic acid by means of zinc dust.

To a solution of haematin in glacial acetic acid zinc dust was added, and the mixture was left in a

See chart X page 105

a stoppered bottle at room temperature for 24 hours. The solution was then deep red in colour. It was filtered, and on spectroscopic examination showed two well marked bands between D and E apparently identical in position with those of metallic haematoporphyrin. After remaining exposed to the air for an hour and half the solution had become much darker red in colour so that it was necessary to dilute the solution with glacial acetic acid before examining it spectroscopically. On spectroscopic examination of the latter solution a faint band was noted in the red between C and D, in addition to the two well marked bands between D and E which have already been described. On the addition of a few drops of hydrochloric acid the bands described disappeared, to be replaced by the bands of haematoporphyrin in acid solution.

Reduction of "the primary product" dissolved in glacial acetic by means of zinc dust.

The filtered solution of the first product of the reduction of haematin in glacial acetic acid was placed in a distillation flask and about two-thirds of the solvent were distilled off under reduced pressure. The pressure was reduced so that the acid

acid rapidly distilled over when the distillation flask was heated on the water bath. The concentrated solution was then dissolved in chloroform. The chloroform solution was next filtered. Part of the pigment remained undissolved in the form of red amorphous granules. The solution in chloroform was washed repeatedly with water in the separating funnel. The chloroform solution was next separated from the aqueous layer and placed in a distillation flask. The pigment residue remaining after distilling off the chloroform was redissolved in glacial acetic acid and reduced by means of zinc dust at a temperature of 60° C. The vessel in which the reduction was carried out was closed by a rubber stopper through which passed a bent outlet tube, which dipped beneath the surface of mercury. Above the mercury a layer of aqueous caustic soda was placed to absorb the vapours of the glacial acetic acid. The following is a rough sketch of the apparatus.

After 8 to 12 hours the solution had acquired a bright green colour and showed two bands, a dark well marked band immediately to the red side of D, and a very faint band immediately on the violet side of D.

An account of the farther investigation of this substance will be given in a later part of this paper.

The haematin employed in the following experiments was prepared in the usual way by solution of the crystals of haemin in dilute caustic soda (1 per cent) and precipitation of the haematin by acidification with acetic acid. The precipitated haematin was filtered off, washed with water, then twice with ether, and dried over sulphuric acid in the desiccator at room temperature.

1. First products of reduction by means of zinc. If a trace of zinc dust be added to a solution of haematin in glacial acetic acid, the lower layers of the solution become bright red or pink in colour; while the upper layers retain the dark red brown colour of an acid haematin solution. On shaking up the fluid it shows the dark red brown colour of an acid haematin solution. After remaining at rest for a short time the solution again acquires its bright red tint. These results suggested that in the first stages of reduction by zinc a pigment was formed which had a great avidity for oxygen and which on oxidation appeared to be reconverted into acid haematin. It has already been seen that even in the cold haematoporphyrin is formed by the reduction of haematin dissolved in glacial acetic by means of zinc. If a substance intermediate

intermediate between haematin and haematoporphyrin be formed it can only be present in the earlier stages of the reduction. In order to ascertain whether an intermediate pigment were formed, it was therefore necessary to obtain a less active reducing agent. The method used to secure this object was to dilute the glacial acetic acid solution with ether. A concentrated solution of haematin in glacial acetic acid was diluted with seven times its volume of ether. The acid ethereal solution was then filtered, and to the filtrate a small quantity of zinc dust was added. The lower layers of the solution immediately became bright red in colour, a little later the whole of the solution had become bright red with the exception of a shallow dark red brown superficial layer. On spectroscopic examination the bright red solution showed a well marked band between D and E. The margin of the band nearest to D was sharply limited while the other margin was shaded. More exact spectroscopic observations were rendered difficult owing to the presence of zinc dust in suspension. On shaking up the solution with air it again became of a dark red brown tint. The solution on being filtered and examined spectroscopically showed only the band of acid haematin in the red. To prevent

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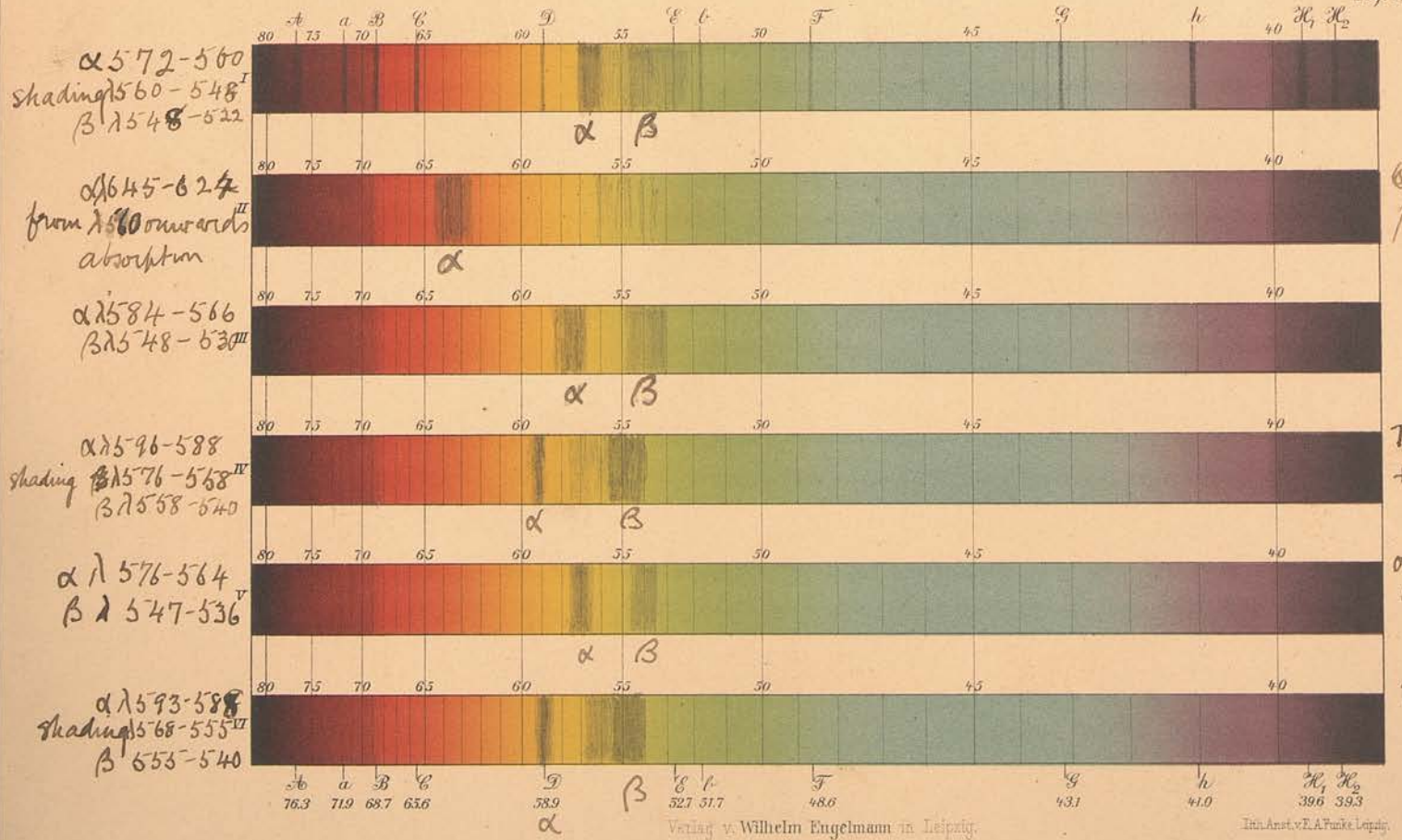

Fig 1.

Fig 2

Fig 3

Fig 4

Fig 5

Fig

prevent the reoxidation of the pigment produced by reduction, the following method was adopted. Through the ethereal solution a stream of carbon dioxide was passed for five minutes, a trace of zinc dust was then added and the stream of carbon dioxide continued for another five minutes. The test tube in which the reduction was carried out was then closed by a tightly fitting rubber stopper, and the solution was examined spectroscopically. It showed spect. chart The α band extends from λ 572 - 560. Towards the red end of the spectrum its margin is sharp and well defined. The area from λ 560 - 548 is shaded. At λ 548 the shading becomes darker and extends to λ 522. No absorption band present in the red. On spectroscopic examination the solution shows therefore two absorption bands α and β connected by a shaded area. Of these α is a much darker band than β. The solution was left in the test tube for 24 hours and on spectroscopic examination still showed the same absorption bands. The solution was then exposed to the air and filtered. It acquired a dark red brown colour and showed spectrum 2 of chart VIII a dark band in the red between C and D extending from λ 645 - 624. The latter is evidently the spectrum of acid haematin. To confirm this, the acid ethereal solution

solution was placed in a porcelain basin and the ether driven off on the water bath. ~~The~~ A solution of the pigment in glacial acetic acid was thus obtained. On dilution with three volumes of water the pigment was partially precipitated. Its precipitation was completed by partial neutralisation of its solution with ammonia. The precipitate was filtered off, dissolved in 1 in 4 ammonia and reduced by means of ammonium sulphide. The solution showed on spectro-scopic examination the absorption bands of haemochromogen in alkaline solution. The first product of the reducing action of zinc on haematin is therefore probably haemochromogen or reduced haematin in acid solution.

A solution of acid haemochromogen was left for 5 days in an acid ethereal solution of the same composition as that first used. There is no evolution of free hydrogen, the hydrogen resulting from the action of acetic acid on the zinc being taken up by the pigment, so that the test tube could be kept tightly stoppered without risk. During the four days the following changes were noted on spectroscopic examination. First the shading between α and β gradually faded. Then the β band appeared to be increasing in intensity becoming at the same time

time narrower and showing better defined margins. The β band becomes gradually shifted neared D. At the end of the ~~fourth~~ fifth day the solution shows the two absorption bands figured in chart.

The α band extends from λ584-566 while the β band extends from λ548-530. The solution was then filtered and exposed to the air. It retained its bright red colour and spectroscopic characters unaltered. One-fifth of its volume of concentrated hydrochloric acid was then added to the solution. On spectroscopic examination after the addition of hydrochloric acid, it showed spectrum chart The α band extends from λ596-588. The region from λ576-558 is faintly shaded and there is a dark band β extending from 558-540. This spectrum is evidently that of a solution of acid haematoporphyrin.

A comparison of the spectrum of the same pigment in ethereal solution with that of metallic haematoporphyrin given in chart VII indicates that the two pigments are identical. chart VIII 7193

In order to ascertain what products were formed subsequent to haematoporphyrin, an acid ethereal solution of haematin to which zinc had been added was kept in a stoppered bottle for seven days. Spectroscopic examination showed that the α and β bands grad-

gradually fading. On the twelfth day the solution had become almost colourless. It had a faint yellow tint. On spectroscopic examination it was found to show no absorption bands. The tube was then unstoppered and the solution was filtered. The colour of the solution soon darkened to an orange and in three hours it had acquired a red colour. On spectroscopic examination it showed two bands α and β having the following positions α λ 576 - 564 β λ547 - 536 (?). Chart VIII. fig. 5.

On adding one-tenth of its volume of concentrated hydrochloric acid the following spectrum was noted fig. 6. α λ 593 - 584, a faintly shaded area from λ 568 - 555 then a distinct band β extending from λ555 - 540. The spectrum of the latter pigment seems to be that of a dilute solution of acid haematoporphyrin while that of the former closely resembles that of metallic haematoporphyrin. In neither solution was a band present in the region of F. The results of these experiments may be summarised in the following scheme.

Haematin dissolved in glacial acetic acid
| 1 vol. Ether 7 vols + Zinc.
Haemochromogen in acid solution.
|
Haematoporphyrin.
|
Chromogen of haematoporphyrin.

If haemin dissolved in acid ether be used for the above experiments instead of haematin, acid haemochromogen is again formed at the first stage of the reduction; but in 24 hours its conversion into haematoporphyrin is completed. This result indicates the marked action of the trace of hydrochloric acid set free from the haemin in accelerating the process of reduction. The previous observers did not obtain haemochromogen amongst the reduction products of acid haematin, probably because in the presence of mineral acid the haemochromogen was converted into haematoporphyrin as rapidly as it was formed.

Reduction of haematin or haemin dissolved in glacial acetic acid by means of Aluminium powder.

Since preliminary experiments had shown that the first pigment obtained by the reduction of haemin by aluminium has the same spectroscopic characters and the same solubilities as that similarly prepared by the reduction of haematin, I afterwards used haemin dissolved in glacial acetic acid instead of haematin. The solubility of haemin in glacial acetic acid is greater than that of haematin. It was mainly for this reason that I used the former.

former.

Four grammes of haemin were dissolved with the aid of heat in 150 ccm. of glacial acetic acid. The solution was then placed in a flask provided with a reflux condenser. 3 gms. of aluminium powder were then added and the mixture was boiled for thirty minutes. In about five minutes the solution had acquired a dark red colour. In about ten minutes it was bright red and the colour remained unaltered on heating for another fifteen minutes. The solution was heated for a longer period than preliminary experiments had indicated to be necessary, in order to ensure complete conversion of the haemin into the first product of the reduction. Preliminary experiments had also shown that if haemin in glacial acetic acid be boiled for an hour with aluminium powder the first formed product undergoes no further change. The bright red solution thus obtained was allowed to cool and was then filtered free from excess of aluminium. The solution was then gradually poured into one litre and a half of a five per cent solution of caustic soda. Part of the pigment separated out from the solution. On gradual addition of strong caustic soda more of the pigment separated out. The addition of caustic soda was continued

continued until the solution became nearly neutral. Finally the solution was rendered strongly alkaline by the gradual addition of 10 per cent sodium carbonate. The pigment had then passed completely into solution. The alkaline solution was heated on the water bath for twenty minutes. The solution was then filtered. The residue on the filter paper was washed with 10 per cent sodium carbonate solution until the filtrate came through colourless. The later washings were thrown away since they contained practically none of the pigment. The yellowish brown residue remaining on the filter paper was washed with water, then with hydrochloric acid (1 in 10). The residue dissolved in the hydrochloric acid to form a light yellow solution. On the addition of potassium ferrocyanide to this solution a voluminous precipitate of prussian blue formed. This indicated that the iron had been split off from the haemin molecule and that the first product of reduction with aluminium was an iron free pigment. The pigment was precipitated from its alkaline solution by acidification with hydrochloric acid. The acid fluid containing the precipitate in suspension was then heated for half an hour on the water bath. The solution was allowed to cool and laid aside for

for twenty-four hours. The supernatant fluid was then decanted from the precipitate, which had settled to the bottom of the vessel, and filtered. The precipitate of pigment was next transferred to the filter, and washed first with water, afterwards with hydrochloric acid (1 in 10) until the acid filtrate no longer gave a reaction for iron. The pigment is very slightly soluble in dilute acids so that during the washing there is very little loss. Finally the precipitate was freed from acid by washing with water. In order to purify the pigment and ensure, if possible complete removal of inorganic salts of iron, it was dissolved in two per cent sodium carbonate, the alkaline solution was filtered, and the pigment again precipitated by the addition of acid. The pigment was finally washed with water, and with ether, in which it is only very slightly soluble, and was then dried over sulphuric acid in the desiccator. A portion, which had been dried at 100° C. was found to be less soluble in the solvents afterwards mentioned, so that I later always dried the pigment at room temperature.

In order to avoid the inconvenience of having to use large quantities of alkali for neutralising the glacial acetic acid, I afterwards adopted the

the following modification of the above method based upon the fact that the pigment which may be termed the primary product of reduction with aluminium is readily soluble in chloroform. The solution of the pigment in glacial acetic acid was mixed with twice its volume of chloroform and the mixed solution was then poured into large excess of water. The chloroform settled to the bottom of the vessel as a dark red solution containing practically all the pigment, the supernatant aqueous layer being almost colourless. The solution of the pigment in chloroform was freed from excess of acid by being repeatedly washed with water in a separating funnel. The solution in chloroform was finally separated from the aqueous layer, and placed in a distillation flask. The pigment residue left on distilling off the chloroform was dissolved in 2 per cent sodium carbonate solution. The subsequent procedure adopted for the separation and purification of the pigment was the same as that already described.

The pigment obtained by this method is readily soluble in dilute alkalies, almost insoluble in dilute aqueous mineral acids. It is readily soluble in chloroform, amyl and ethyl alcohols and phenol (in the heat), less readily soluble in acetone, toluene

toluene and aniline, and very slightly soluble in ether, acetic ether and petroleum.

I have made numerous attempts to obtain the pigment in a crystalline form; but as yet without result. Since the pigment is almost insoluble in dilute mineral acids the method employed by Nencki for the crystallisation of the hydrochloride of haematoporphyrin is not available. For crystallisation of the pigment the following methods were tried. Saturated solutions of the pigment in chloroform, amyl, and ethyl alcohols, acetone, and aniline were allowed to slowly evaporate over sulphuric acid in the desiccator. The pigment separated from all of these solvents in the form of amorphous granules. I then tried to crystallise it from mixed solvents. From its solutions in chloroform, phenol, ethyl and amyl alcohols, the pigment may be more or less completely precipitated by the addition of ether, acetic ether or petroleum ether. By the gradual addition of the latter solvents to solutions of the pigment in chloroform, ethyl and amyl alcohols, I tried to obtain a crystalline precipitate; but so far all the precipitates thus obtained were found on microscopical examination to be amorphous.

The following method was adopted to determine whether the primary product was iron free. 0.4 gms. of the pigment were incinerated with 10 cc. of concentrated sulphuric acid plus 3 gms. of potassium sulphate. When the mixture had become transparent and almost colourless, it was allowed to cool and then diluted with water so as to make up 250 ccm. 5 ccm. of 1 in 20 hydrogen peroxide were then added so as to oxidise any ferrous salt present. The solution was then heated on the water bath for half an hour. After cooling potassium ferrocyanide was added. The solution became blue; but even after standing for 24 hours only a slight deposit of prussian blue was formed. Iron was therefore only present in traces.

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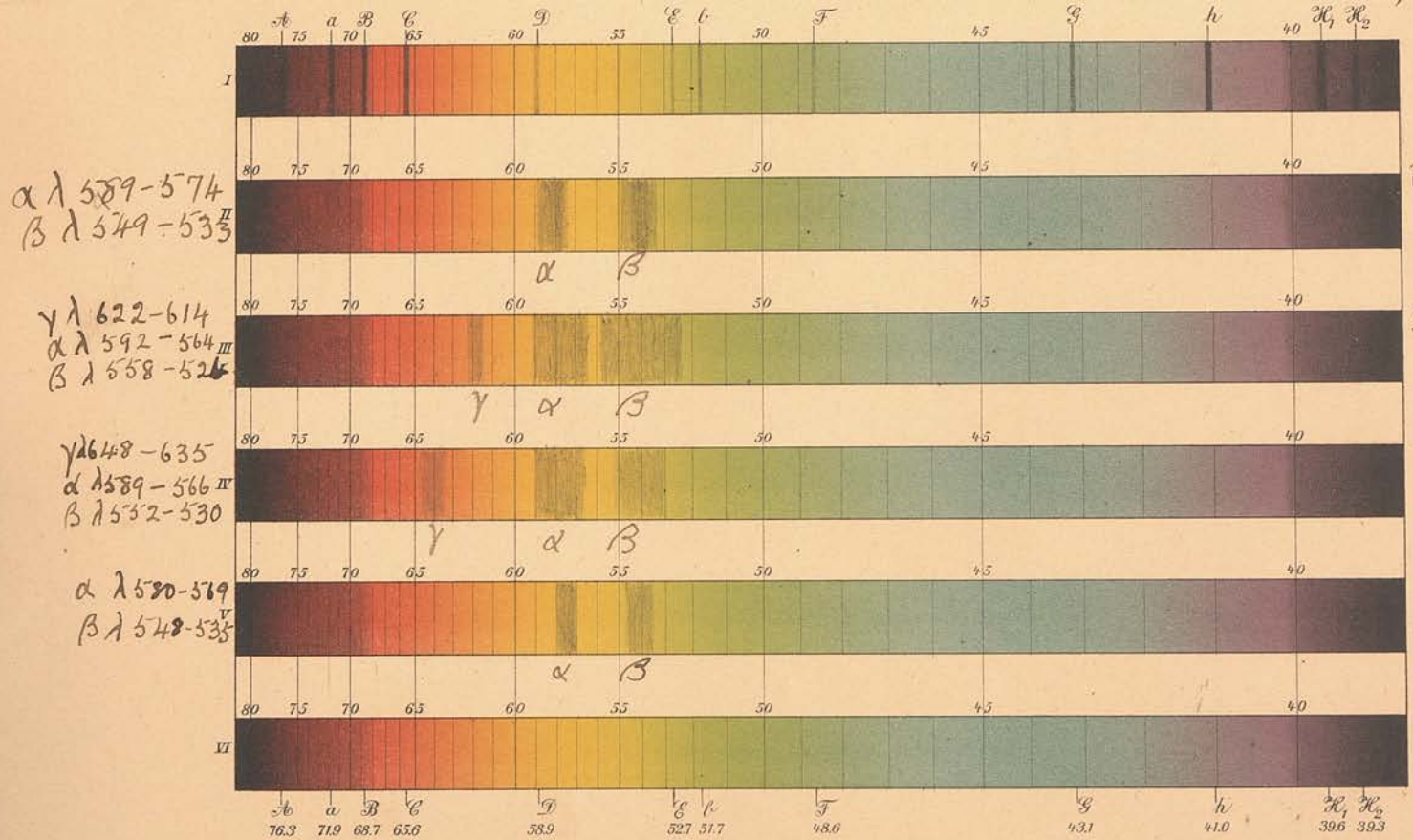

Pigment dissolved in acetic acid. Fig

Same in a thicker layer. Fig

The same pigment in Na_2CO_3 sol^n Fig

The pigment in glacial acetic + HCl. Fig

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On spectroscopical examination of a solution of this pigment in glacial acetic two bands α and β were seen situated between D and E. The α band extends from λ 589 - λ 574, while the B band extends from λ 549 - λ 533. The same solution was then examined in a thicker layer and a faint band γ was then seen in the red between C and D lying nearer D and extending from λ 622 - λ 614. The α and β bands are broader and darker than when examined in a thinner layer, α extending from λ 592 - 564 and β from λ 558 - λ 524. (see figures 1 and 2 spectroscopic chart IX)

The same pigment dissolved in sodium carbonate shows the α and β bands practically unchanged in position and character, α extending from λ 589 - 566 and B extending from λ 552 - 530. It will be noted, however, that the band γ in the red has become displaced towards C now extending from λ 648 - λ 635. The presence of a band in the red is therefore not due to an admixture of the primary reduction product with some unchanged haematin. For, if the band γ in the red had been due to haematin, it would have been found near C in acid solution while in alkaline solution it would have moved up to D. Figure 4 of spectroscopic chart IX shows the spectroscopic characters of a dilute solution of the same pigment

pigment in three volumes of glacial acetic acid plus one volume of concentrated hydrochloric acid. If allowance be made for the fact that the solution is more dilute and the bands α and β are therefore fainter, the position of the bands appears to be the same as in alkaline and organic acid solutions. The α band extends from λ580 - λ569 while the β band extends from λ548 - λ535. The γ band is no longer to be seen. Probably this is due to the fact that the solution examined was a dilute one.

I have not yet made any exact spectrophotometric observations with regard to the relative intensity of the bands; but I have obtained the following results by dilution of an alkaline solution. On dilution the γ band disappears first. Both the α and β bands become narrower and fainter; but still remain perceptible even in very dilute solutions showing only a faint red colour. Ultimately only the α band is seen distinctly, the B band being however still perceptible as a faint shading. The order of intensity of the bands is therefore α most intense, then B, and finally γ the latter being a much less intense

intense band than either α or B.

A comparison of the spectrum of this pigment with that of metallic haematoporphyrin shown in figure 4 chart VII shows that the α and β bands are identical in position and character with the corresponding bands of metallic haematoporphyrin. It differs however from metallic haematoporphyrin in not showing the spectrum of acid haematoporphyrin, when dissolved in a solvent containing excess of free mineral acid, and also in showing a faint band in the orange. In the constancy in position and character of the α and β bands in alkaline neutral, and in acid solution it closely resembles the form of metallic haematoporphyrin described by Saillet. I have, however, heated a dilute solution of the pigment in hydrochloric acid (1 in 4) without producing any alteration in its spectroscopic characters; while under the same conditions the form of haematoporphyrin described by Saillet is converted into acid haematoporphyrin.

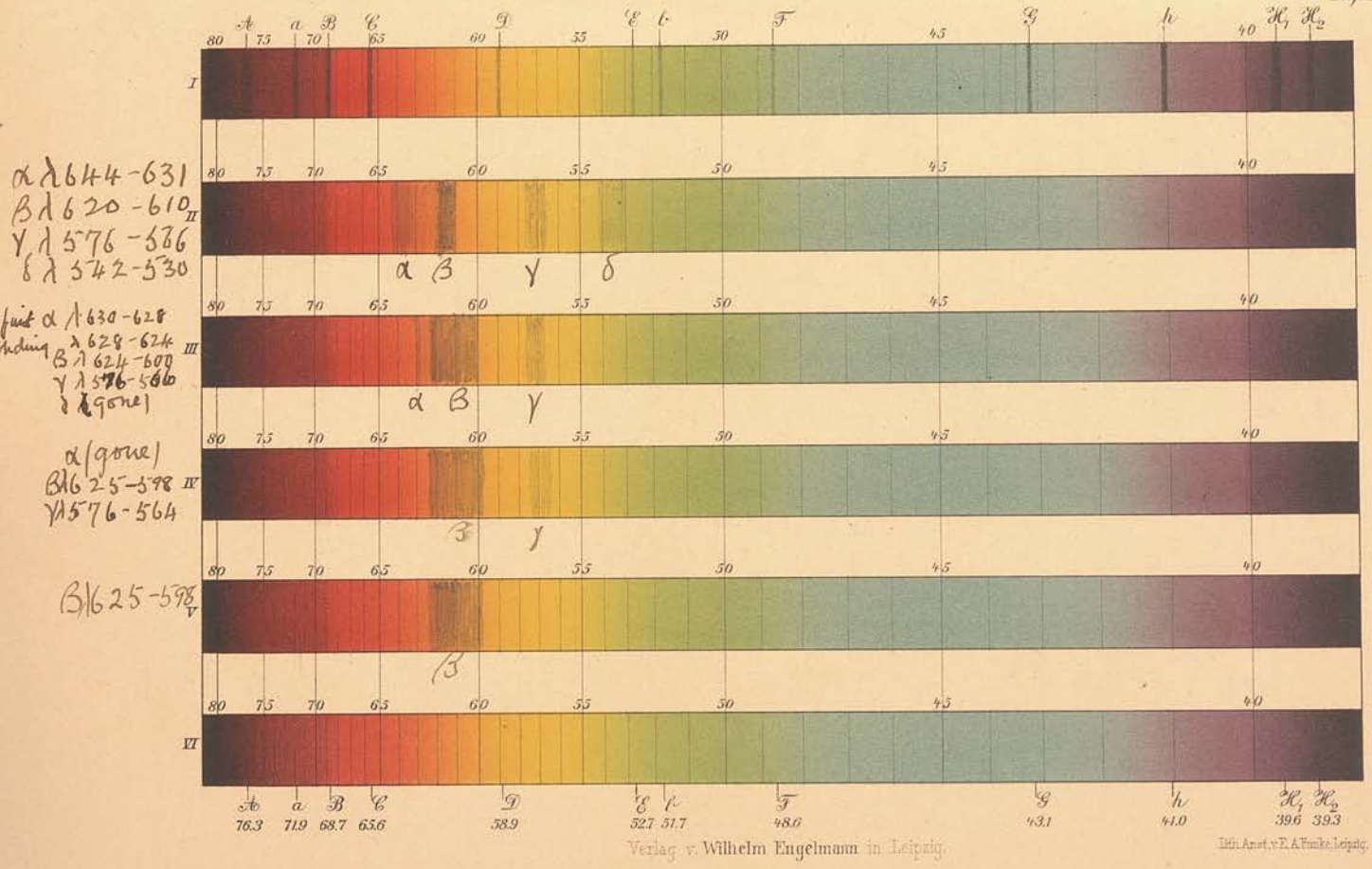
It also closely resembles the so-called hexahydrohaematoporphyrin obtained by Nencki and Sieber

Sieber, the band between b and F present in the latter being absent. This band, as has already been seen, is probably due to admixture with a pigment resembling urobilin. The pigment described by Nencki and Sieber differs from it in being insoluble in alkalies. The fact that it is practically insoluble in aqueous solutions of mineral acids, while readily soluble in alkalies, and that in solution in glacial acetic plus hydrochloric acid it does not show the spectrum of acid haematoporphyrin indicates that it is a form of haematoporphyrin which has lost the power of playing the part of a base although still retaining its acid character. I thought that the loss of basic character might be due to acetylisation of the pyrrol group upon the presence of which the basic character of haematoporphyrin probably depends. This seemed possible in view of the fact that pyrrol on being treated with acetic anhydride yields n-acetyl pyrrol. In order to saponify this derivative, if present, I dissolved the pigment in ten per cent caustic soda and boiled the solution for half an hour. The pigment was then precipitated by the addition of hydrochloric acid to its alkaline solution, filtered off, and dissolved in alcohol acidified with hydrochloric acid. Spectroscopic

Spectroscopic examination of the solution in acid alcohol still showed the two bands α and B unaltered in position or character. By this method the pigment had, therefore, not been converted into acid haematoporphyrin.

Reduction of the primary product dissolved in glacial acetic acid by means of zinc.

Three grammes of the primary product of the reduction of haemin by means of aluminium were dissolved in 200 ccm. of glacial acetic acid. The solution was heated to 60° C. and about ten grammes of zinc were gradually added. The reduction takes place most readily if the total amount of zinc be not added at one time, but in quantities of about 2 grammes at a time. The vessel in which the reduction was carried out was kept stoppered, as the hydrogen generated is taken up by the pigment. The following spectroscopic changes were noted. The α and β bands of the primary product gradually become narrower and less distinct. Simultaneously the band in the orange whose centre is about λ616 becomes more intense. Still later the band in the orange is well marked extending from λ620-λ610. A little later a band is seen lying still nearer the red end of the spectrum.



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spectrum. The centre of the latter band lies about λ642. The latter band gradually increases in intensity and extends from λ644- λ631. Of the two bands between C and D that extending from λ620-610 is much the darker. A little later the area between these two bands becomes shaded. At this stage the solution has acquired a bright green tint and the B band of the primary reduction product has disappeared. The α band has become replaced by a faint shading extending from λ576-566. These changes may be best followed by a reference to figures 1 to 4 of chart X. At a still later stage only one band is present between C and D and a very faint band is present immediately to the violet side of D. Ultimately the latter band disappears. The solution requires to remain about seven hours at 60° C. in order to reach this stage. Figure 2 spectral chart XI shows the following absorption bands--a band extending from λ625-598 and very faint shading from λ576-564. The green pigment showing these spectroscopic characters readily undergoes a change on exposure to the air in acetic acid solution. The change is indicated by a rapid alteration in the colour of the solution which commences in its upper layers and gradually extends downwards. The solution, on

on exposure to the air, first becomes olive green and then yellowish brown. The fact that this change takes place first in the upper layers of the fluid indicates that it is due to oxidation by the air. On adding more zinc, and heating the mixture, the colour of the solution again becomes green and shows the spectroscopic characters already described. The unstable character of this pigment renders its isolation and purification difficult. It is readily soluble in chloroform and when obtained in a solution in chloroform retains its spectroscopic characters practically unaltered. It is difficult however to prevent the oxidation of the pigment during its transference from acetic acid into chloroform. I was not at first completely successful in preventing oxidation and obtained a pigment differing in spectroscopic characters from that originally present.

For preventing its oxidation, I at first used apparatus similar to that employed by Hoppe Seyler for the investigation of the decomposition products of haemoglobin in the absence of oxygen; but found it very difficult to carry through the transference of the pigment from glacial acetic acid into chloroform and then the washing of the chloroform solution with water so as to remove the acetic acid mixed

mixed with it, without the entrance of air. I ultimately employed the following simple method. The glacial acetic acid solution of the pigment was mixed with twice its volume of chloroform, and filtered into 2 litres of a one per cent solution of sodium carbonate. The evolution of carbon dioxide produced prevents reoxidation. When carbon dioxide ceases to be freely evolved, the supernatant aqueous layer is poured off and replaced by five per cent sodium carbonate, a stronger solution of the alkali being used in the later stages. The pigment at first dissolves completely in the chloroform forming a dark green solution; but if the washing with sodium carbonate solution be too prolonged, part of the pigment commences to pass from the chloroform into the aqueous layer. The washing with the alkaline solution is stopped when carbon dioxide ceases to be evolved. The chloroform solution is then siphoned off, placed in a separating funnel and repeatedly washed with distilled water. The solution of the pigment in chloroform is finally separated from the aqueous layer. Figure 3/12 spectroscopic chart XI shows the absorption bands present. In a shallow layer only one band is present, situated between C and D extending from λ 626-610. If this spectrum be compared

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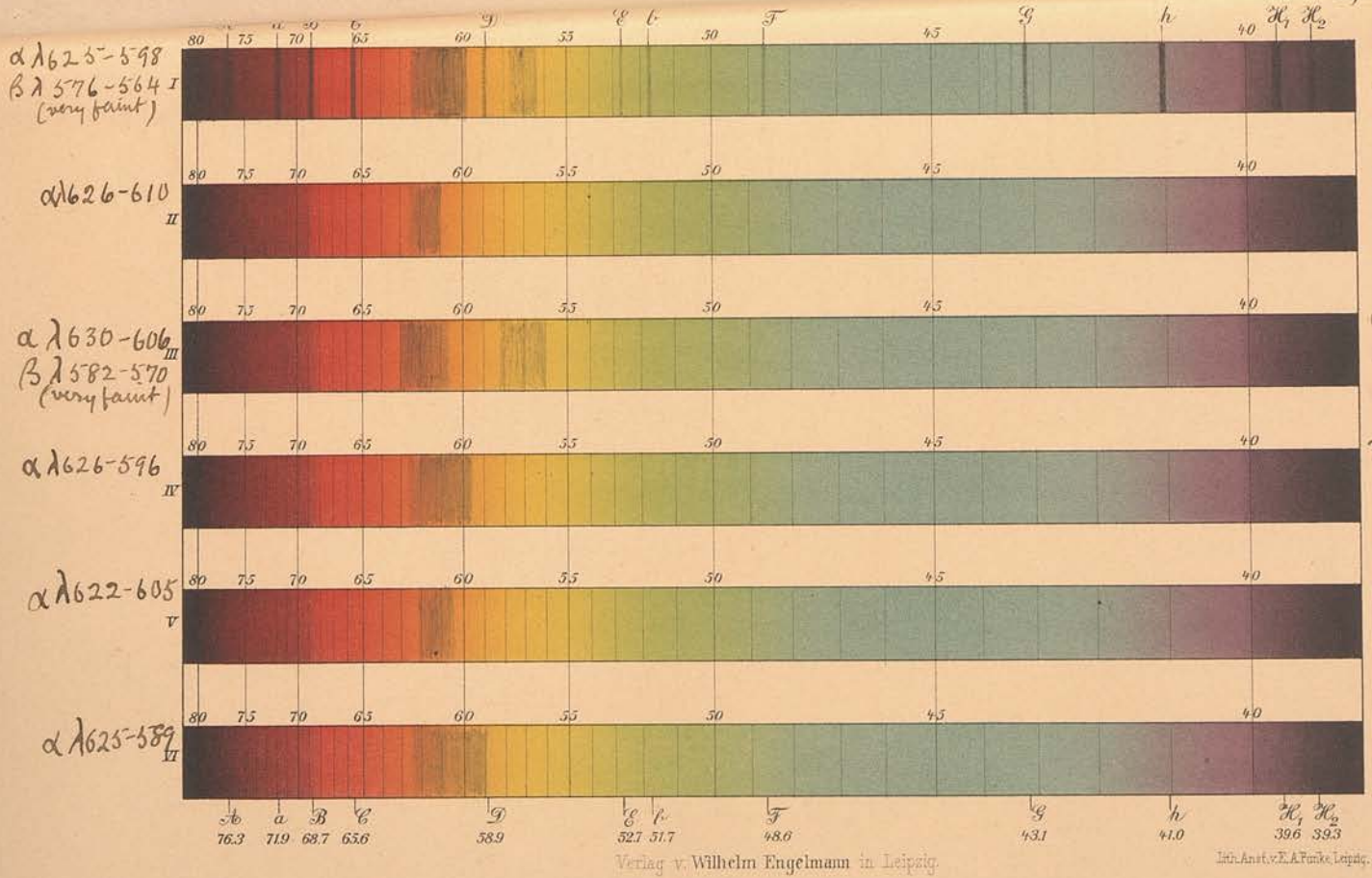

in glacial acetic (slightly oxidised) — Fig 1.

Same in chloroform — Fig 2.

chloroform sol^n in a thicker layer

Pigment (not oxidised) in glacial acetic acid. — Fig 3

dissolved in sodium carbonate — Fig 4

Product of reduction in acetic anhydride — Fig 5

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compared with that of the original solution (Fig. 3 chart XI) they will be seen to be very similar. In the solution in glacial acetic acid the absorption band extends a little farther towards the violet end of the spectrum. Fig. 2 chart XI shows the same solution examined in a thicker layer. The pigment thus obtained retained its spectroscopic characters unaltered for several months when dissolved in chloroform. It appears therefore to be much more resistant to the oxidising action of the air when dissolved in chloroform than when in solution in glacial acetic acid. Figure spectroscopic chart shows the pigment dissolved in two per cent sodium carbonate solution. It will be noted that the position of the band in the red varies only slightly according to the reaction of the solvent. The band β in the yellow extending from λ 582-570 is relatively much fainter and is only seen well on examination either of a concentrated solution or of more dilute solution in a thick layer.

Unless one adopts the above described precautions to prevent the oxidation of the pigment during its transference from glacial acetic acid into chloroform, one obtains pigments having the spectroscopic characters shown in figures 1 & 3 chart. XII It will be noted

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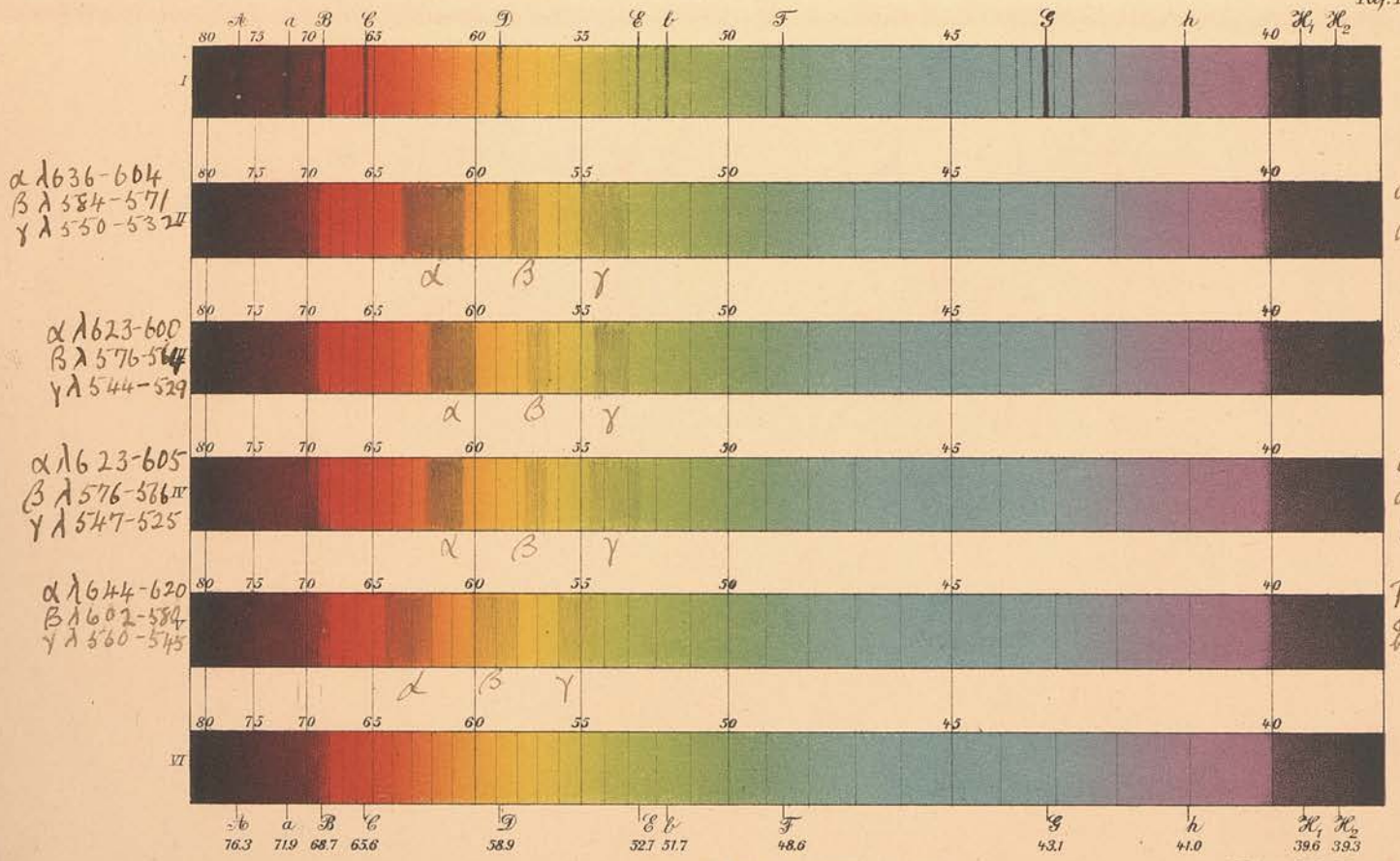

Fig 1

Fig 2

Fig 3

Fig 4

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noted that in addition to the band in the red, two relatively faint bands appear between D and E. These are identical in position with the ~~A~~ and B bands of the primary reduction product, and are probably due to the fact that part of the green pigment, which may be termed the secondary reduction product, has become reconverted into the primary reduction product by oxidation. Figures 163 chart XII show the partially oxidised pigment in neutral, alkaline and mineral acid solution. It will be noted that the position and character of the bands are retained unaltered. Examined in a shallow layer by transmitted light the partially oxidised pigment appears greenish blue. In a deeper layer it appears of a violet tint. By reflected light the solution appears blue with a very well marked red fluorescence.

For the purification of the secondary reduction product the following method was employed. Its solution in chloroform was placed in a distillation flask and the residue left on distilling off the chloroform was dissolved in 2 per cent sodium hydrate. The alkaline solution was then filtered and freed from zinc by a current of sulphuretted hydrogen. The solution was again filtered and the pigment precipitated by acidification with hydro-

hydrochloric acid. The precipitate was transferred to a filter washed first with 1 in 10 hydrochloric acid and with water until the acid had been removed. Finally the pigment was dissolved in one per cent sodium carbonate, the solution filtered and purified by reprecipitation with hydrochloric acid. After being washed with 1 in 10 hydrochloric acid, with water and with ether, the pigment was dried over sulphuric acid in the desiccator.

The pigment so obtained forms a dark green amorphous powder, which is readily soluble in dilute alkalies, insoluble in dilute mineral acids. It dissolves readily in chloroform, amyl alcohol, ethyl alcohol, aniline, pyridine and dimethylaniline. It is almost insoluble in ether, acetic ether and petroleum ether. I tried without success to obtain the pigment in the crystalline form by concentrating its saturated solutions in the first six solvents over sulphuric acid in the desiccator. From a solution in amyl alcohol which was saturated at 100° C. the pigment separated out in minute green crystals mixed however with a large proportion of the pigment in an amorphous form. From its solutions in amyl and ethyl alcohols it may be partially precipitated by the addition of ether. I have not however been

been able to obtain it in the crystalline form by this means. The precipitate, which separates out, is found on microscopical examination to consist of minute green granules showing no trace of crystalline structure.

I also tried to obtain the secondary product in a form more resistant to the oxidising action of the air by the use of acetic anhydride plus sodium acetate as a solvent for the primary product instead of glacial acetic acid. 0.5 grammes of the primary reduction product were dissolved in 50 cc. of acetic anhydride containing 4 grammes of dry fused sodium acetate. 3 grammes of zinc dust were then gradually added to the mixture, and the solution was boiled in a flask with reflux condenser. After about 10 minutes boiling the solution had become bright green in colour. It was boiled for a farther 10 minutes, and was then slightly cooled. A small portion of the fluid was filtered while hot. The solution was at first bright green in colour and showed on spectroscopic examination the same characters as the colouring matter obtained by reduction of the primary in glacial acetic acid solution. On exposure to the air the solution gradually underwent a change in colour which commenced in the upper layers of the solution.

solution. These became first olive green and then yellowish brown. The secondary product of reduction obtained under these conditions shows as great a liability to undergo oxidation when in acid solution as that prepared by reduction in glacial acetic acid solution. The pigment prepared by reduction in acetic anhydride ~~by~~ plus sodium acetate solution resembles that prepared by the first method in its spectroscopic characters, but differs from it in being much less soluble in aqueous alkalies. The fact that it is less soluble in alkali probably indicates that it is a partially acetylised derivative of the pigment first described.

Products of the reduction of the secondary dissolved in glacial acetic acid on more prolonged heating with zinc dust.

1 gm. of the primary reduction product was dissolved with the aid of heat in 100 ccm. of glacial acetic acid. To this solution four grammes of zinc dust were added and the mixture was boiled in a flask with reflux condenser for two hours. In about 10 minutes the solution had become bright green in colour. On farther heating for half an hour, the solution had become lighter green in colour. After being boiled for three hours the solution had a light

light green colour with a slight yellow tint. On spectroscopic examination the solution showed a dark band in the red extending from λ 624- λ 608 and also a faint band in the dark green of the spectrum extending from a short distance to the violet side of b nearly to F. (The position of the latter band was determined by means of a small direct vision ~~of~~ spectroscope.)

On more prolonged boiling the band between C and D gradually became less distinct while the band between b and F increased in intensity. On spectroscopic examination of the solution after it had been boiled for eight hours, the band between C and D was still visible; while that situated between b and F was well marked. The solution was now reddish brown in colour and showed a faint green fluorescence. The changes in the spectroscopic characters of the solution appeared to indicate that the secondary reduction product was undergoing a slow conversion into a pigment resembling urobilin.

This conversion may be much accelerated by the addition of hydrochloric acid. A solution of the secondary product of reduction in glacial acetic acid was mixed with one-ninth its volume of concentrated hydrochloric acid, zinc dust being afterwards added. There was at first a rapid evolution of hydrogen, the solution at the same time undergoing a change of colour from green to greenish brown. When the evolution of hydrogen had almost ceased, the solution was examined spectroscopically. It showed one faint band between C and D and a well marked band between b and F. The solution was heated on the water bath for half an hour and then filtered.

filtered. The filtrate was light yellow in colour showing no trace of green fluorescence. On spectroscopic examination one well defined band was perceptible filling up about two-thirds of the space between b and F and extending a little distance beyond F. The band between C and D had completely disappeared. The solution was then left exposed to the air for three days, and still showed the same spectroscopic characters.

A solution of the secondary reduction product dissolved in five per cent caustic soda was also boiled with zinc dust. Spectroscopic changes similar to those above described were noted. The band between C and D gradually fades and a band in the green between b and F gradually becomes more distinct and ultimately is alone present.

The pigment finally obtained evidently closely resembles urobilin in its spectroscopic characters.

The course of the reduction of haemin dissolved in glacial acetic and reduced first by aluminium and then by zinc may therefore be represented by the following scheme.

scheme.

Aluminium plus glacial acetic acid → Haemin → Primary reduction product

Zinc plus glacial acetic acid → Secondary reduction product

Zinc plus glacial acetic acid 9 parts, HCl 1 part → Urobilin

The evidence with regard to the chemical nature of the secondary reduction product which I have so far obtained is insufficient to establish its identity with any of the known animal pigments; but some of the chemical and physical characters of the pigment suggest a relationship between it and the bile pigments. Haematoporphyrin and bilirubin are isomeric and apparently yield on reduction the same final product both within the organism and under the influence of chemical reducing agents. During the reduction of bilirubin, Disqué (33) and Eichholz (17) have obtained reduction products intermediate between Bilirubin and Urobilin. In the first stage the hydrobilirubin described by Maly is formed, while later on normal urobilin is obtained as a final product. From my own observations it appears that a product of reduction may also be obtained intermediate between haematoporphyrin and urobilin. The question

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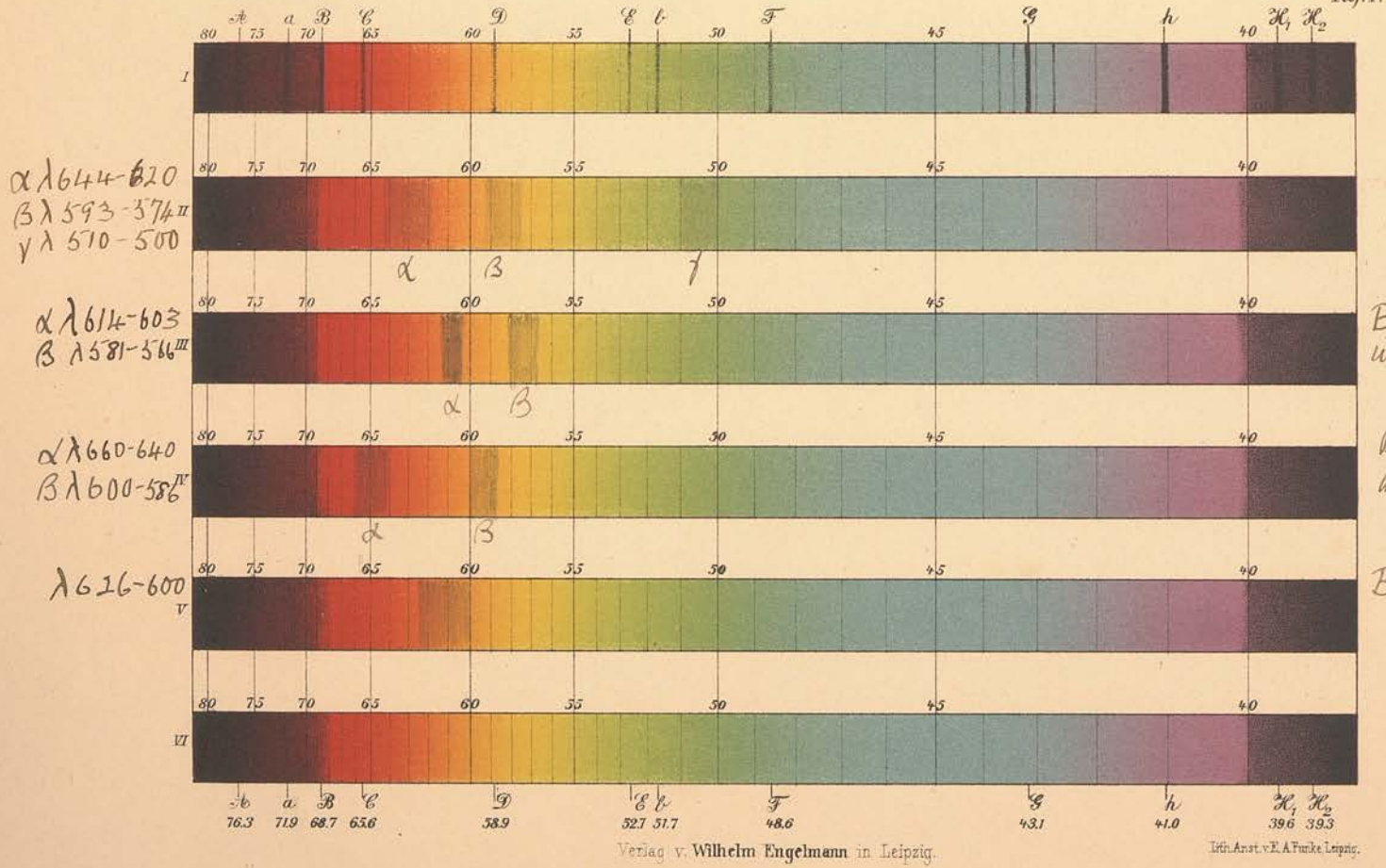

Biliverdin in acid solution

Biliverdin in alkaline solution

Bile of pig

Fig 1.

Fig 2

Fig 3 *

Fig 4

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* The band between b + F seen in solutions of biliverdin is probably due to admixture with cholestelin and has therefore not been depicted.

question therefore arises as to whether there is any resemblance between the pigment which I have obtained and that described by Eichholz (17) as an intermediate product in the reduction of bilirubin. (Chart XIII Fig 1) The pigments, however, seem to have only superficial resemblances. A solution of the partially oxidised secondary reduction product shows a well marked red fluorescence. Alkaline solutions of the substance obtained by Disqué also showed a red fluorescence; but in spectroscopic characters the two pigments are quite distinct. The spectroscopic characters of the secondary reduction product when in acid solution bear a close resemblance to those of bilicyanin; but in alkaline solution bilicyanin (23) shows a spectrum quite distinct from that of the secondary reduction product. (Chart XIII (5) Figs. 2 and 3)

Prior to the work of Eichholz, MacMunn also studied the reduction products of bilirubin and came to the conclusion that the hydrobilirubin described by Maly (30, 31 1932) is an intermediate stage. The spectroscopic characters of the bile vary in different animals. Since bilirubin and biliverdin show no absorption bands, the bands noted on spectroscopic examination of bile excreted by different animals must be due to other pigments which are probably either products of the oxidation or of the reduction of bilirubin.

bilirubin. The bile of the pig shows, on spectroscopic examination, a band extending from about λ626-λ600. (Chart XIII Fig 4) This band is very similar in position to that of "the secondary reduction product." It would be unwise to attach too great importance to this similarity in spectroscopic character without farther chemical evidence. (344) The fact that Jaffé and MacMunn have obtained urobilin from the bile of various animals would indicate that in seeking for the pigments to which the bile of different animals owes its spectroscopic characters, one must bear in mind the fact that these pigments may be either products of the reduction or of the oxidation of bilirubin. Whether the pigment to which the bile of the pig owes its spectroscopic character belongs to the former or to the latter class is unknown.

Before concluding this account of the products of the reduction of haematin or haemin in organic acid solutions it is necessary to consider whether by prolonged reduction with aluminium or zinc, one can obtain a pigment resembling urobilin. In order to reach this stage, I found that it was necessary to prolong the boiling for several days, about 40 hours in the case of zinc, and about 60 in the case of aluminium. The length of time seems to vary

vary according to the concentration of the haematin solution. It has already been seen that in order to rapidly reach the final stage the addition of mineral acids is necessary. The product obtained by very prolonged reduction of haematin dissolved in glacial acetic acid by means of aluminium forms at first a light yellow solution showing a faint green fluorescence. The solution acquired a reddish brown tint on exposure to the air, and the green fluorescence became more marked. The pigment was then transferred to chloroform. Its solution in chloroform was also reddish brown, showed a well marked green fluorescence and on spectroscopic examination a well defined band lying between b and F. After the solution in chloroform had been kept for a month, the fluorescence had disappeared, although the spectroscopic characters of the pigment had remained unaltered. The residue obtained by evaporating off the chloroform was dissolved in ammoniacal alcohol, to which zinc chloride was afterwards added; but even under these conditions the green fluorescence did not reappear.

Reduction of haematin in alkaline solution by means of zinc dust and aluminium.

By the reduction of haematin in alkaline solution,

solution, I obtained results similar to those recorded by previous observers. I studied somewhat fully the conditions under which haemochromogen, showing the typical spectroscopic characters, is formed. For the reduction in alkaline solution, zinc is to be preferred to aluminium. The aluminium hydrate formed during the later stages of the reduction forms a precipitate with the pigments. If a solution of haematin in caustic soda be heated with zinc or aluminium, no apparent change at first takes place. If on the other hand a solution of haematin in ammonia be heated with aluminium or zinc, it almost immediately acquires a cherry red tint, and shows the spectrum of alkaline haemochromogen. The influence of the presence of proteid was shown in the following way: To a solution of haematin in 4 per cent caustic soda alkali albumin and glucose were added. The mixture on being heated acquired the bright red tint and showed the spectroscopic characters of a solution of alkaline haemochromogen. The same change was brought about by the use of aluminium as a reducing agent instead of glucose. The above test may be used to determine whether a given specimen of haematin be free from proteid or no. Tested in this way the haematin prepared by the method given

given on page appeared to be free from proteid.

Products of the distillation of haemin and haematin with zinc dust.

In view of the fact that Hoppe Seyler obtained a substance giving the pyrrol reaction by the distillation of a reduction product of haematin with zinc dust, I thought that it would be important to find out whether the substance giving the pyrrol reaction bears any resemblance to the haemopyrrol obtained by Nencki and Zaleski (page of this thesis). A preliminary experiment proved that, when one part of haemin was intimately mixed with ten parts of zinc dust, and the mixture then heated, a colourless vapour came off which gave a deep red colour to a pine wood match dipped in concentrated hydrochloric acid. On strongly heating, reddish vapours were also observed.

For the distillation the following method was employed. 3 gms. of haemin were thoroughly mixed with 30 gms. of zinc dust and the mixture then placed in a combustion tube, through which a current of carbon dioxide previously dried by passing through sulphuric acid and anhydrous calcium chloride was then passed. The opposite end of the combustion tube was connected by means of an adapter with

with three wash bottles, the first containing chloroform, the second ether and the third a dilute solution of hydrochloric acid. The current of carbon dioxide was passed through the tube for half an hour. The tube was then gradually heated to a dull red heat. A substance distilled over which condensed in colourless drops on the sides of the adapter. In about an hour the distillation of this substance had ceased. The combustion tube was then allowed to cool, while a slow current of carbon dioxide was passed through the apparatus.

The chloroform and ether in the wash bottles were at first colourless. On exposure to the air they acquired a very faint yellow tint. The solutions were then concentrated on the water bath, and as the concentration proceeded they became first yellow, then yellowish brown, and showed a faint green fluorescence. On spectroscopical examination the solution showed a well marked band in the green between b and F (see fig. 3 chart W) Part of the chloroform solution was evaporated to dryness. The residue, which was reddish brown in colour, was dissolved in ammoniacal alcohol. The alcoholic solution was light yellow in colour and on addition of zinc chloride the solution showed a green fluorescence *

* The dilute aqueous solution of hydrochloric acid was at first colourless. It after on exposure to the air it became light yellow. The solution was too dilute to shown any absorption bands

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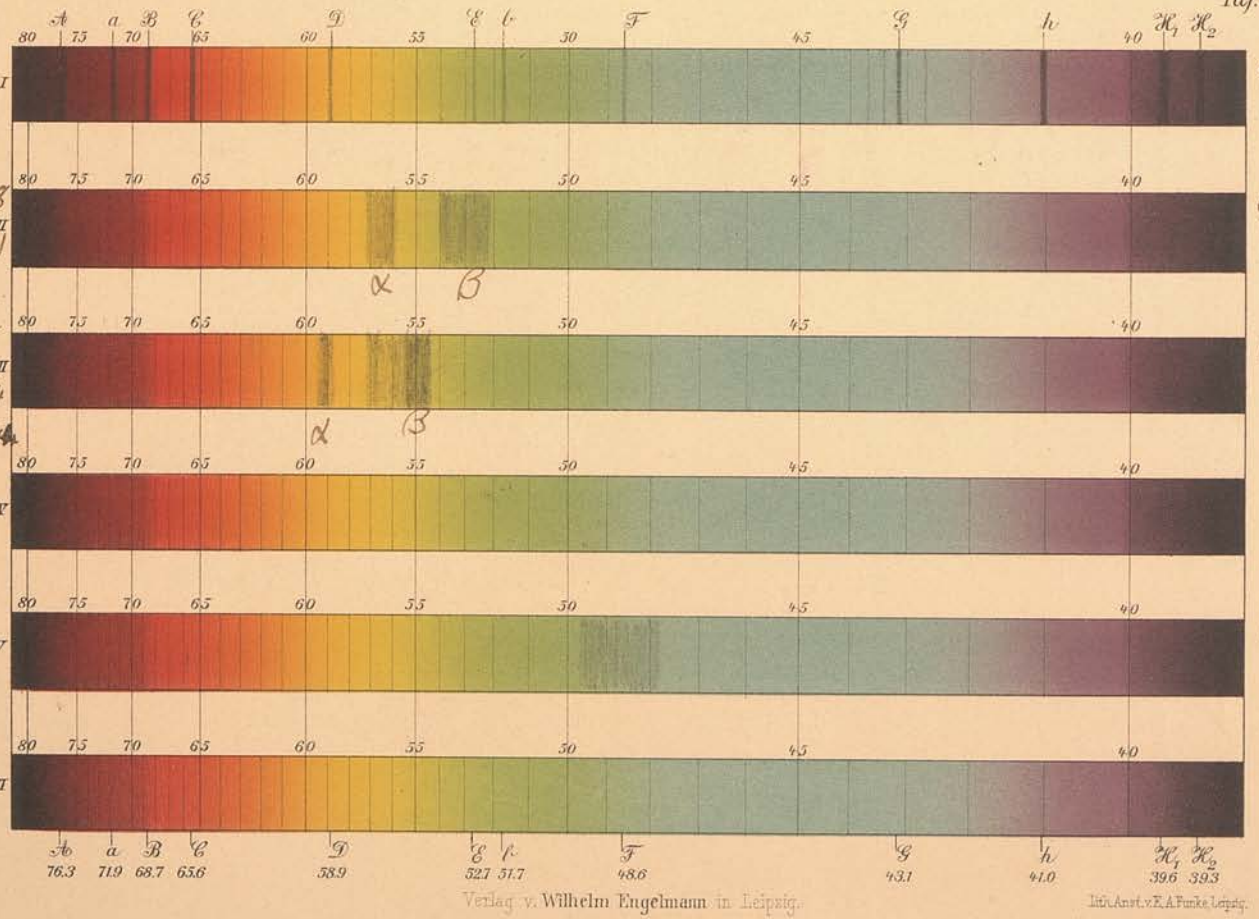

Fig 1.

Fig 1.

Fig 2

Fig 3.

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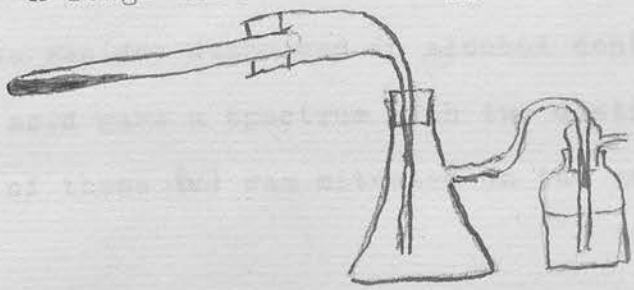
fluorescence and the band in the green became better defined and shifted slightly towards the red end of the spectrum. The volatile product obtained by the above described method appears therefore to be a chromogen of urobilin. I have not yet obtained it in a sufficient quantity for a more thorough chemical examination. In its conversion into a substance resembling urobilin it behaves similarly to the haemopyrrol obtained by Nencki. On being kept for a fortnight it was noted that the solution of the pigment in chloroform had lost its green fluorescence and that the band between b and F was fainter.

For the further study of the products obtained by the distillation of haemin with zinc dust I employed the following method. 1.5 gms. of haemin were intimately mixed with 10 gms. of zinc dust, and 4 gms. of calcium carbonate. The mixture was then placed in a hard glass tube connected by means of an adapter with a filtration flask, the lateral tube of which was connected with a Drechsel's wash bottle containing chloroform. A rough sketch of the apparatus is given below.



The calcium carbonate was mixed with the zinc in order to obtain a rapid current of carbon dioxide which might carry with it the earlier products of the reducing action of the zinc before they had undergone farther reduction. The part of the tube containing the mixture was then strongly heated. A red coloured vapour formed which filled the tube and adapter and also passed into the receiving flask. This red vapour soon condensed in the form of red drops on the walls of the adapter and flask. When it ceased to be evolved the flame was withdrawn. As the apparatus cooled some chloroform passed from the wash bottle into the receiving flask and dissolved the red pigment forming a bright red solution. After the apparatus had cooled, the receiving flask and adapter were washed out with chloroform. The solution of the pigment in chloroform showed three bands α extending from λ 572-558 and β extending from λ 540-521. Chart XIV Fig 1 The position of these bands is almost identical with that of the β and γ bands of alkaline haematoporphyrin. Another band was present, situated between b and F. Part of the chloroform solution was evaporated to dryness. The residue dissolved in alcohol containing hydrochloric acid gave a spectrum with two distinct bands. One of these (α) was situated on the red

red side of D extending from λ595-λ588. The second band consisted of two parts--a lightly shaded part from λ572-λ554 and a dark part from λ554-λ540 (Figure 2 chart XIV).

Its spectrum is evidently identical with that of acid haematoporphyrin. The pigment residue left on evaporation of a solution of the pigment in chloroform was found to be insoluble in aqueous alkalies. In its insolubility in aqueous alkalies this pigment resembles phylloporphyrin. I have not yet had time for its fuller investigation.

In forming a sublimate on being heated the pigment differs from haematoporphyrin. On referring to Marchlewski's monograph (34) on chlorophyll, I find no statements made with regard to the behaviour of phylloporphyrin when heated.

The study of the products of the decomposition of haematin not only throws light upon the probable stages in the Katabolism of blood pigment; but, when considered in relation to E. Fischer's (21) discovery of α pyrrolidine carboxylic acid amongst the products of the hydrolytic decomposition of proteids by dilute acids and trypsin, it also adds to our knowledge with regard to the probable precursors of the

the blood pigment. It seems probable that the molecular group in proteid which yields pyrrolidine carboxylic acid is also the group which is utilised in the synthesis of haematin by the organism.
Until the constitutions of haematin and haematoporphyrin are known, conjectures with regard to the probable stages of this synthesis are of little value; but it is noteworthy that the ease with which pyrrol and its derivatives undergo polymerisation under the influence of acids and alkalies eminently fits them for utilisation by the organism in synthetic processes.

The following equations may be given showing the change undergone by pyrrol under the influence of acids and heat

$$(C_4H_5N)_3 = \begin{matrix} CH=CH-C^{H}-CH \\ | \qquad\qquad \| \quad \| \\ CH=CH-C \quad C-H \\ \qquad\qquad\quad \backslash \quad / \\ \qquad\qquad\quad NH \end{matrix} + C_4H_3N + NH_3$$

(Tripyrrol) (Indol) (Pyrrol)

If the nature of the chemical change involved in the conformation of urobilin from its chromogen (haemopyrrol) is still unknown. An enumeration of the conditions favouring the transformation is given on page IX

The fact that several alkaloids also contain the pyrrol (e.g., nicotine and strychnine) and pyrrolidin (e.g., atropine and cocaine) groups, suggests the possibility that in the animal organism also they may be the source of some of the known active principles. In this connection it is interesting to note that Abel [124] has found a pyrrol derivative amongst

amongst the products of the distillation of "epinephrin" with zinc dust, and that he has also obtained skatol, which belongs to the benzopyrrol group, from it on heating with caustic potash. Certain preliminary experiments by Abel also suggest that uroerythrin may possibly be a derivative of epinephrin.

Ultra violet

~~Photographic~~ spectrum of the pigments.

I have as yet only carried out a few preliminary experiments with the object of ascertaining whether any of the pigments above described show bands in the ultra violet region.

The spectrum obtained from the arc light by means of a flint glass prison having a refracting angle of 60° was projected on a screen and the ultra violet region examined with the aid of a fluorescent screen coated with barium or potassium platmocyanide. I did not observe any absorption bands in the ultra violet region of the spectrum of the primary reduction product. The secondary reduction product showed a faint band situated about the junction of the visible and invisible violet filling in the space between two of the bands due to the volatilisation of the carbons of the arc light.

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